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Does Caffeine Induce Dominant Lethal Mutations in Mice?*

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Summary. The problem of mutagenic activity of caffeine (1-3-7-trimethylxanthine) in man becomes more and more urgent because it is: first, one of the most frequently used stimulants in beverages and drugs and; second, recently several contradictory reports have been published [37, 29, 1, 13] which were followed up in public with great interest. We, therefore, tested the spermatogenesis of mice with the method of induction of dominant lethal mutations [42] in order to find a mutagenic effect, and by fractionating the spermatogenesis in eight breeding groups to detect some sensitive stages after a single treatment with a high caffeine dose. The animals used were of the C3H inbred strain of mice, the dose just tolerated by these animals was 0,25 g caffeine/kg body weight. Two experiments were carried out under same conditions with 29 males treated and 10 males in the control group. 373 pregnant females fertilized by the treated males showed among their 3495 implants 15% dead implantations while 204 females fertilized by the control males had 2139 implants, 13,6% of which died during pregnancy. This slight difference does not indicate any mutagenic function of caffeine on the spermatogenesis of mice as a whole. The tested stages of spermatogenesis revealed no sensitivity. A slight increase in pseudopregnancy ($P \approx 0{,}007$) could be observed, which might be physiologically caused. Copulation frequency was considerably decreased, especially in the first week after treatment.

Until now it seemed impossible to decide whether caffeine (1-3-7-trimethylxanthine) is mutagenic in man or not. Experiments carried out with various organisms, such as bacteria [12, 14, 16] and Ascomycetes [15, 51], plants [23–27, 31, 35], HeLa- and leucocyte cultures [37–39, 41] and in some cases even with Drosophila [1, 32, 33, 40] have shown the mutagenic activity of caffeine. However, in each system these findings lead to a different hypothesis concerning the mechanism of caffeine mutagenicity. BIESELE et al. (1952) [6] thought that perhaps caffeine was directly incorporated into DNA as a base analog. However, working with E. coli, KOCH (1956) [28] showed that labeled caffeine does not combine with DNA. A more indirect mechanism seemed plausible when bacteriologists found that caffeine enhances the mutagenic activity of UV light by seemingly reducing the efficiency of the repair mechanism [9–14, 20, 21, 30, 44, 45]. In plants, on the other hand, KIHLMAN (1952) [25] discovered the "radiomimetic effect" of caffeine, — so called because the chromosome-breaking effects in Vicia faba root tips resembled in several details those induced by x-rays. A further addition to these divergent lines of evidence was made by cytological experiments [37] with HeLa- and leucocyte cultures, which lead to the conclusion that caffeine induces chromosome breaks mainly during DNA replication. An estimation of caffeine-induced mutation rate in man, based upon the results of these in vitro experiments with human cells, was found to range in the magnitude of natural mutation

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rates [38]. However, the results of in vivo tests with mice so far published [1, 2, 7, 31] and in some cases those with Drosophila were negative. The only direct investigation carried out with regard to humans was an enquiry among students as to the partaking of coffee by their parents and as to the number of their sisters and brothers. The result was also negative.

It is obvious that the information so far is very fragmentary and often contradictory. However, the possible mutagenic effect of caffeine is of great interest because it is a common constituent of drugs, and moreover, it is ingested with coffee and other beverages. We suspect that it is quickly metabolized in mice as in other mammals [5]. For man AXELROD and REICHENTHAL (1953) gave a metabolic half life of 3.5 hrs. More important, caffeine is dissolved in the tissue fluid and spread throughout the body within a short period of time. Investigations by GOLDSTEIN and WARREN (1962) [18] found the same concentrations of caffeine in the plasma and the gonadal tissue — even in the fetus — 30 min after a dose of 1 g caffeine sodium benzoate (574 mg caffeine) was administered.

To settle the question of caffeine mutagenicity in man more satisfactorily, it seemed necessary to enlarge the number of in vivo investigations in mice, in order to have data, which could be extrapolated to the situation in man. We, therefore, investigated the effect of a single treatment, with a high caffeine dose, on different stages of spermatogenesis, to see if an increase in induced dominant lethal mutations occured, and if perhaps some stages of spermatogenesis were more sensitive to caffeine than others.

Material and Method

C3H inbred male mice aged 10 weeks ± 2 days were given a single dose of 0.25 g caffeine/kg body weight, in 1 ml 0.9% NaCl-solution by intraperitoneal injection. Control animals received 1 ml NaCl-solution. In the first experiment, (A) 15 males were treated with caffeine, and 5 males treated with isotonic saline served as controls. One year later, a second experiment (B) was carried out with 10 males in the same season and under the same conditions: 14 of them treated, the remaining 5 serving as controls. 1 day after treatment, each male was mated with 3 virgin C3H inbred females aged from 10 to 11 weeks. The animals remained together for 1 week. This was repeated seven times, in order to fractionate the spermatogenesis of the males into 8 series, corresponding to the different stages [13, 42]. The methods used in the dissection of the females, in the counting of the corpora lutea graviditatis and of the living and dead implantations, as well as in the mode of evaluation, are described by RÖHRBORN (1968).

The females were dissected after 14.5 to 16.5 days of pregnancy. The preimplantational egg loss was determined by the ratio of implants to corpora lutea graviditatis $\left(\frac{\text{c. l.} - \text{impl.}}{\text{c. l.}} \cdot 100\right)$, while the postimplantational egg loss was summarized from three groups of dead embryos. The so-called deciduomata, brownish necrotic remainders of implants, which died at about the time of implantation or short after, consist mainly of maternal tissue. The early stages, on the other hand, died a little later and revealed a dark red placenta and residues of fetal tissue. The third group, late implantational death, can be well separated from both mentioned above by identifying the anlagen of the eyes.

As statistical method for judging the difference between two observed rates (preimplantational egg loss etc.) or the heterogeneity in a series of such rates, the χ^2-test was used; in the latter case the χ^2-value was calculated by the simple formula of BRAND-SNEDECOR (WEBER, 1961)

$$\chi^2 = \frac{N^2}{K(N-K)}\left(\sum_{i=1}^{r} \frac{k_i^2}{n_i} - \frac{K^2}{N}\right) \quad (r-1 \text{ degrees of freedom})$$

where k_i/n_i $(i = 1, 2 \ldots r)$ are the observed rates and $K = \Sigma k_i$, $N = \Sigma n_i$. We considered values of P (level of significance) greater than 0.05 as criterion for no real difference resp. no real heterogeneity. P-values less than 0.01 as suspective and those under 0.0027 as evidential for a real difference resp. real heterogeneity. In comparing the observed rate in an experiment with that in controls a one side test with levels of significance halved can be used, because an experimental rate lower than the control rate has no real meaning.

Results

The animals showed only weak signs of toxicity after one time treatment with 0.25 g caffeine/kg body weight. Although GUMMEL and KIESE (1938) gave a LD_{50} average of 0.23 g caffeine/kg body weight, none of our males died. At a dose of

Table 1. *Pre- and postimplantational egg loss in experiment A after one dose of 0.25 g caffeine/kg body weight, injected intraperitonealy in 1 ml isotonic NaCl-solution. Control animals received 1 ml of the solvent only*

series	treated ♂♂		fertile ♀♀		n c.l.		n implants		% PRE		% D.I.	
	V	K	V	K	V	K	V	K	V	K	V	K
I	15	5	17	15	167	149	141	124	15.6	16.8	19.1	15.3
II	15	5	26	14	268	152	217	123	19.0	19.1	11.5	11.4
III	15	5	30	13	300	138	264	122	12.0	11.6	15.2	12.3
IV	15	5	29	12	296	122	267	110	9,8	9,8	15.7	14.5
V	15	5	27	13	293	140	254	109	13.3	22.1	15.4	19.3
VI	15	5	29	13	312	142	257	131	17.6	7.7	17.1	15.3
VII	15	5	30	12	314	126	278	109	11.5	13.5	14.7	15.6
VIII	15	5	29	13	323	140	288	123	10.8	12.1	18.1	9.0
Σ	15	5	217	105	2273	1109	1966	951	13.5	14.2	15.8	14.0

c.l. = corpora lutea graviditatis. — PRE = preimplantative egg loss. — D.I. = dead implants or postimplantative egg loss.

Table 2. *Pre- and postimplantational egg loss in experiment B after one dose of 0.25 g caffeine/kg body weight, injected intraperitonealy in 1 ml isotonic NaCl-solution. Control animals received 1 ml of the solvent only*

series	treated ♂♂		fertile ♀♀		n c.l.		n implants		% PRE		% D.I.	
	V	K	V	K	V	K	V	K	V	K	V	K
I	14	5	12	13	127	136	114	119	10.2	12.5	8.8	14.3
II	14	5	17	12	200	122	174	111	14.9	9.0	16.1	10.8
III	14	5	22	14	243	151	215	126	11.5	16.6	16.3	11.1
IV	14	5	20	11	221	130	197	116	10.9	10.8	11.2	9.5
V	14	5	28	10	296	95	273	88	7.8	7.4	15.0	14.8
VI	14	5	19	12	211	142	181	120	14.2	15.5	22.0	17.5
VII	14	5	19	10	197	103	185	89	6.1	13.6	10.8	14.6
VIII	14	5	19	14	208	131	190	123	8.7	18.5	9.5	13.0
Σ	14	5	156	96	1703	1030	1529	892	10.2	13.4	14.0	13.1

c.l. = corpora lutea graviditatis. — PRE = preimplantative egg loss. — D.I. = dead implants or postimplantative egg loss.

0.3 kg/g 6 out of 8 males died within 30 min post injection. Thus the dose of 0.25 kg/g is just tolerated by the C3H inbred mice.

The values of pre- and postimplantational egg loss are given in Table 1 (experiment A) and Table 2 (experiment B). The sum totals of all 8 series in each experiment showed no differences in the rates of dead implantations with or without treatment. In experiment A the χ^2 was 1.58 (P = 0.2), in experiment B χ^2 was

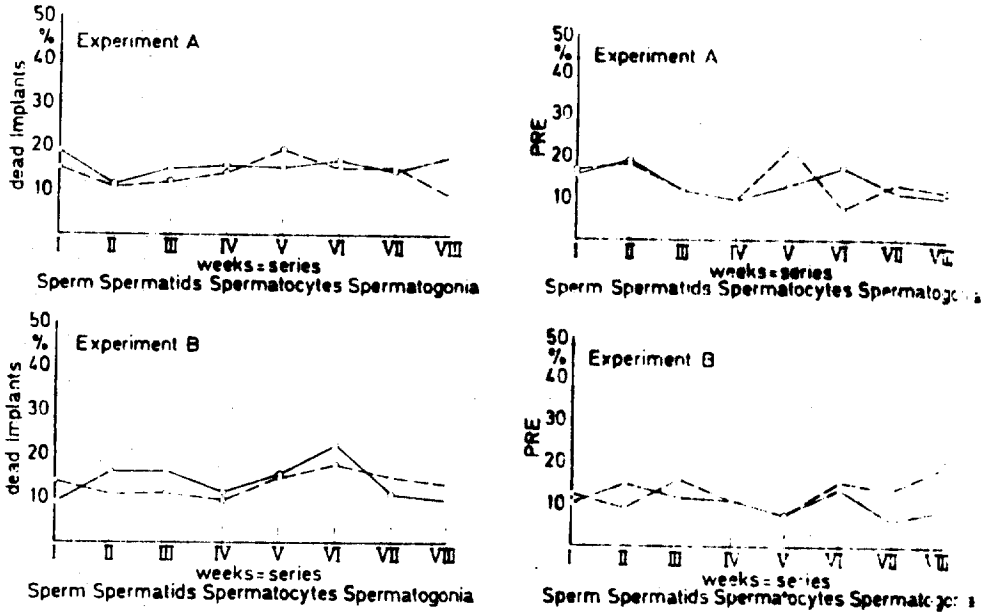

Fig. 1 Fig. 2

Fig. 1 Postimplantational egg loss after i.p. injection of 0.25 g/kg caffeine in C3H inbred male mice. treated group, control group

Fig. 2 Preimplantational egg loss after i.p. injection of 0.25 g/kg caffeine in C3H inbred male mice. treated group, control group

0.7 (P = 0.3). The preimplantational egg loss was even slightly lower after caffeine treatment (exp. A: 13.5%, with caffeine 14.2% in the control, exp. B: 10.2%, with caffeine, 13.4% in the control)

Fig. 1 gives the distribution of dead implantation rates found in the eight series of both experiments, the curves of preimplantational egg loss are shown in Fig. 2. All curves lie in about the same range. In some cases the preimplantational egg loss varies between treatment and control, i.e. series VI of experiment A 17.6% and the corresponding control only 7.7%, or series II of experiment B has 11.9% while the control has only 9.0%. This pattern, however, is not consistant, for the values often vary in the other direction, i.e. series V of experiment A is 13.3%, while the control is 22.1%; series VII of experiment B has only 6.1% while the control is 13.6%, also series VIII of experiment B has 8.7% and the control is 18.5%. These fluctuations seem to indicate that the differences are accidental and are no true manifestations of stage sensitivity

Testing the homogeneity of the postimplantational egg loss rates in the two experiments with the χ^2-method revealed no heterogeneity in experiment A ($P > 0.5$) but heterogeneity in experiment B ($P \approx 0.0052$, or $P < 0.0027$ one sided). The P-values in χ^2 testing the differences between experimental and control rates in the single series of the two experiments are shown in Table 3 and 4. Only one series (VIII) in experiment A has such a low P-value ($P \approx 0.01$ one

Table 3. *P-values for the postimplantational egg loss of each series in experiment A*

Series	n implants		n D.I.		% D.I.		Probability
	V	K	V	K	V	K	
I	141	124	27	19	19.1	16.3	> 0.44
II	217	123	25	14	11.5	11.4	> 0.96
III	264	122	40	15	16.2	12.3	> 0.49
IV	265	110	42	16	15.7	14.5	> 0.75
V	254	109	38	21	15.4	19.3	> 0.32
VI	257	131	41	20	17.1	16.3	> 0.78
VII	278	109	41	17	14.1	15.6	> 0.8
VIII	288	123	52	11	18.1	9.0	< 0.02
Σ	1966	951	310	133	15.8	14.0	> 0.2

Table 4. *P-values for the postimplantational egg loss of each series in experiment B*

Series	n implants		n D.I.		% D.I.		Probability
	V	K	V	K	V	K	
I	114	119	10	17	8.8	14.3	> 0.17
II	174	111	28	18	16.1	10.8	~ 0.2
III	215	126	36	14	16.3	11.1	> 0.17
IV	197	116	22	11	11.2	9.5	~ 0.75
V	273	88	41	13	15.0	14.8	> 0.95
VI	181	120	40	21	22.0	17.5	> 0.3
VII	185	89	20	13	10.8	14.6	> 0.32
VIII	190	123	13	16	9.5	13.0	> 0.3
Σ	1629	892	214	117	14.0	13.1	> 0.3

sided) that a difference could be suspected but the result might be accidental. Furthermore series VI and VII show no differences between treatment and control groups even though they also represent the spermatogonial fraction of the spermatogenesis. If caffeine has a mutagenic action on spermatogonia it should be manifest in all three series.

In considering the heterogeneity of experiment B, it is notable that the P-values are not significant in any series. But if we compare the values of the series against the mean value of the controls we find a significant difference in series VI, in which, as mentioned above, cells treated as spermatogonia fertilize the egg cells. Thus there are two weak references that the spermatogonia are influenced by caffeine.

In order not to overlook a slight mutagenic activity, an attempt was made to pool the test material to enlarge the nummerical values. Before doing this the homogeneity of the control values of both experiments was tested with the formula of BRANDT-SNEDECOR. The results — experiment A: $\chi^2_7 = 6.56$, $P = 0.45$; experiment B: $\chi^2_7 = 4.91$, $P = 0.66$ — showed no heterogeneity. The examination of each series of the material as a whole revealed no significant differences in the

Table 5. *Pre- and postimplantational egg loss in the summary of experiment A and B*

series	treated ♂♂		fertile ♀♀		n c.l.		n implants		% PRE		% D.I.	
	V	K	V	K	V	K	V	K	V	K	V	K
I	29	10	29	28	294	285	255	243	13.3	14.7	14.5	14.8
II	29	10	43	26	468	275	391	234	16.5	14.6	13.6	11.1
III	29	10	52	27	543	299	479	248	11.8	14.2	15.7	11.7
IV	29	10	49	23	517	252	464	226	10.3	10.3	13.8	11.9
V	29	10	55	23	589	235	527	197	10.5	16.2	15.2	17.3
VI	29	10	48	25	523	294	438	251	16.3	11.6	19.2	16.3
VII	29	10	49	22	511	229	463	198	9.4	13.5	13.2	15.2
VIII	29	10	48	27	531	291	476	246	10.0	15.5	14.6	11.0
Σ	29	10	373	201	3976	2139	3495	1843	12.1	13.8	15.0	13.6

c.l. = corpora lutea graviditatis. — PRE = preimplantative egg loss. — D.I. = dead implants or postimplantative egg loss.

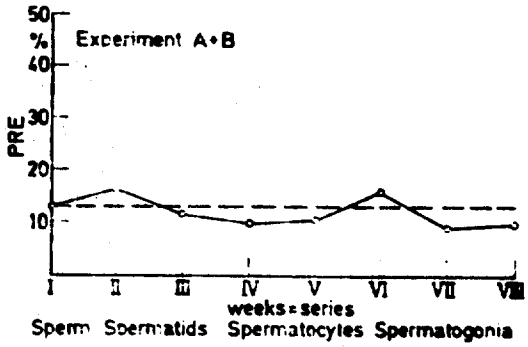

Fig. 3

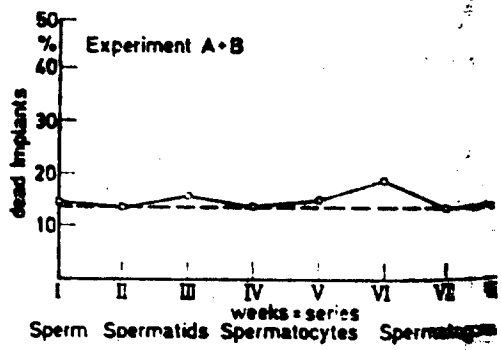

Fig. 4

Fig. 3. Preimplantational egg loss as the sum total of both experiments. ——— treated group; — — — mean value of the controls

Fig. 4. Postimplantational egg loss as the sum total of both experiments. ——— treated group; — — — mean value of the controls

pre- or postimplantational egg loss (Table 5). Again several values of the treated group lie under the mean value of the controls in the case of the preimplantational egg loss (Fig. 3). Most values obtained from dead implants in the treated group lie slightly above the mean value of the controls (Fig. 4). In series VI, there is a significant difference between the treated group and the mean value of the

controls, that is P = 0.0027. But compared with the control of the same series no heterogeneity is to be seen (Table 6). The series VII and VIII revealed no differences either when compared with their own controls or when compared with the mean value of the controls. This could be explained if all embryos of series VI were attacked by a greater number of exogenous influences. Thus the higher rate of dead implants is seen in the control as well as in the treated group, and there

Table 6. *P-values of the postimplantational egg loss in each series of experiment A and B*

series	n implants		n D.I.		% D.I.		Probability
	V	K	V	K	V	K	
I	225	243	37	36	14.5	14.8	> 0.94
II	391	234	53	26	13.6	11.1	> 0.32
III	479	248	75	29	15.7	11.7	> 0.14
IV	464	226	64	27	13.8	11.9	> 0.5
V	527	197	80	34	15.2	17.3	> 0.5
VI	438	251	84	41	19.2	16.3	> 0.35
VII	463	198	61	30	15.2	15.2	~0.5
VIII	478	246	70	27	14.6	11.0	> 0.16
Σ	3495	1843	524	250	15.0	13.6	> 0.34

Table 7. *Fertility reduction in C3H male mice after one dose of 0.25 g caffeine per kg body weight, injected intraperitonealy in 1 ml isotonic NaCl-solution. Control animals received 1 ml of the solvent only. The data derived from both experiments*

series	n implants/♀		fertile ♀♀ % vag. plugs		χ^2	Probability
	V	K	V	K		
I	8.9	8.9	79.4	95.8	3.2	> 0.08
II	9.2	9.0	90.7	91.7	0.02	> 0.88
III	9.3	9.2	84.6	95.5	1.70	< 0.2
IV	9.5	9.8	87.5	88.0	0.004	> 0.98
V	9.5	8.7	83.3	91.3	0.85	~0.35
VI	9.4	10.0	69.5	88.5	3.5	~0.07
VII	9.4	9.1	84.3	90.5	0.5	~0.48
VIII	9.9	9.4	84.0	89.3	0.4	> 0.5
Σ	9.4	9.3	82.7	91.2	7.55	< 0.007

is no real mutagenic influence. This phenomenon shows the necessity of sufficiant controls, especially for the C3H inbred strain of mice (see RÖHRBORN, 1968).

Then an attempt was made to estimate the reduction of fertility. The number of implants per female was the same in the treated and in the control group over the whole series of the summarized experiments (Table 7). The estimation of pseudopregnant females was based on the numerical difference between the vaginal plugs found and the number of pregnant females. Although the %-ratio of real pregnancy to the vaginal plugs found was slightly decreased in the series of the

treated group the differences were not significant. However, the summarized values from all series showed a low P-value of 0.007 and so the influence of caffeine on the enlargement of pseudopregnancy can not entirely be excluded.

Furthermore, in the sum total of all series of both experiments there was a significant difference in copulation frequency after caffeine treatment (P = 0.0001). Table 8 shows that series I and VIII, and also to a smaller extent series II, are

Table 8. *Reduction of copulation frequency after treatment of C3H inbred male mice with one dose of 0.25 g caffeine/kg body weight injected intraperitoneally in 1 ml isotonic NaCl-solution. Control animals received 1 ml of the solvent only. The data derived from both experiments*

series	n mated ♂ ♂		n c.l. ♂ ♂		% c.l. ♂ ♂		Probability
	V	K	V	K	V	K	
I	87	30	34	24	39.1	80.0	0.0001
II	84	30	43	24	51.2	80.0	0.008
III	84	30	52	22	61.9	73.3	> 0.26
IV	84	30	56	25	66.7	83.3	0.056
V	92	32	60	23	65.2	71.9	0.5
VI	95	30	59	26	62.1	86.7	0.025
VII	95	30	51	21	53.7	70.0	0.12
VIII	93	30	50	28	53.8	93.3	0.0001
Σ	703	242	405	193	57.6	77.8	< 0.0001

primarily responsible for this value. This, however, may be entirely due to the effect of caffeine on the central nervous system, which could then in turn reduce the frequency of copulation — an explanation without any genetic implication.

Discussion

The estimation of caffeine-induced mutation rate in man is based on the experimental results in cell cultures obtained with doses 1000 times higher than normally expected in human tissue in vivo [18], and are extrapolated linearly to lower doses which never had been tested [37]. Furthermore, the break-inducing effects in vitro and in vivo are not necessarily the same. For this reason, a careful examination of the supposed mutagenic danger of caffeine was undertaken in mammals in vivo. With C3H inbred mice mutagenic effects, measured in terms of pre- and postimplantational egg loss, were not found after a single treatment with a high caffeine dose. Although the values of preimplantational egg loss differed in some cases between treated and control animals, this difference was probably accidental, due to the influence of such factors as manner of ovary preparation and estimation of corpora lutea graviditatis. The more crucial criterion is the postimplantational egg loss which showed no increase, either in each individual spermatogenic stage or in the experiment as a whole.

However, these negative results could not exclude the possibility that a weak mutagenic effect of caffeine might have been overlooked. In testing the difference between the rates observed in samples of two populations (population of treated mice and control population), two types of error can be made: first, to postulate

a difference between the true rates in the two populations which, in reality, is not present; second, to accept equality of the true rates in a case of an only slight but real difference (VOGEL, 1967) [46]. This second type of error is the dangerous one in our case. So we tried to establish the validity of the negative results by a method described by VOGEL and STROBEL (1958) [47]. If we denote:

p_0, p_1 = true rates of dead implantations in controls resp. experiment,

n_0, n_1 = total number of implantations in controls resp. experiment,

k_0, k_1 = number of dead implantations in controls resp. experiment,

$K = k_0 + k_1$, $t = k_1/k_0$,

$\chi^2_{.1} = 2.706$ = 10% significance point of χ^2 with 1 df., formula [8] of these authors gives an upper 95% confidence limit R_U for the ratio $R = p_1/p_0$ of the true rates of dead implantations:

$$R_U = \frac{n_0}{n_1}\left[t + \frac{(1+t)^2}{2}\,\frac{\chi^2_{0.1}}{K} + \sqrt{t(1+t)^2\,\frac{\chi^2_{0.1}}{K} + \frac{(1+t)^4}{4}\,\frac{\chi^4_{0.1}}{K^2}}\right]$$

In our experiment this confidence limit R_U had the value 1.25, meaning that with probability 95% the true rate of dead implantations in the experiment is at most 1.25 times higher than the true control rate. In other words, an average increase of 25% in the total number of dead implantations under the conditions of our experiments can still be in agreement with our results. So our conclusion is statistically sound.

Using the number of implants per female as a criterion, no reduction in fertility was found. The slight increase in pseudopregnancy observed among the test group as compared with the control, though not significant in a strict sense, cannot be totally ignored. However, since caffeine did not show a clear mutagenic effect via the induction of dominant lethal mutations, it is unlikely that the increase in pseudopregnancy is genetically caused. More likely, it is due to physiological changes of the sperm, perhaps a reduction of sperm mobility or deformation of the sperm head (teratospermia) so that they are unable to penetrate the egg membrane.

CATTANACH (1964) reported a reduction of implants per female after 6 week-treatment of male mice with 0.3% caffeine in the drinking water. He also did not believe this effect to be genetically caused, but postulated a reduction in meiosis frequency resulting in oligospermia. In our laboratory further investigations of this problem are going on.

Finally, the observed reduction of the copulation frequency is perhaps due to a physiological effect of caffeine on the central nervous system.

As mentioned in the introduction, the experiments published so far with caffeine treatment of mice gave negative results. We received a personal communication from KUHLMANN et al. (1969) concerning a test series in which he found a considerable increase in the number of dominant lethal mutations after long term treatment of male C57 Bl mice with caffeine (0.025—0.85%) in the drinking water. However, he worked not only with a smaller number of treated animals, but also with fewer controls. Furthermore, the mode of evaluation differed in some respect to the one we used: i.e. we would not include females with only dead implantations and no living embryos into the estimation of dominant lethal

mutations because the physiological situation in the maternal uterus may contribute to the destruction of all embryos. We, on the other hand, did not find any such case. May be, these phenomenons observed by KUHLMANN are due to some infect which remained undetected and only simulated a mutagenic effect (see RÖHRBORN, 1968).

The same data, which were already reported on the meeting of the Deutsche Gesellschaft für Anthropologie und Humangenetik in Königstein 1967, were now published as a part of a review about experiments with caffeine on human cell cultures, Drosophila and mice by KUHLMANN et al. 1969 [29]. The authors however, don't mention the cases of total implant mortality in some females.

The largest experiments with chronical administration of caffeine solutions as drinking water given for a total period of 245 days were undertaken by RÖHRBORN (1969) [43]. He mainly used C3H inbred mice and (101 × C3H) F_1 hybrids and treated the males with a low dose (0.0023%) which corresponds to three cups of coffee per day for a normal person, and also with higher doses up to 0.5%. No increase in the induction of dominant lethal mutations could be seen in his experiments. So, despite the above mentioned positive results of KUHLMANN, with our methods, we could not find any mutagenic effect of caffeine, and would prefer not to condemn the substance.

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24. The Problem of Caffeine Mutagenicity[1, 2]

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With 1 Figure

Caffeine (1-3-7-Trimethylxanthine) is suspect as a mutagen because it might be incorporated as a base analog into the DNA because of its purine nature. Since caffeine is found in most soft drinks and coffee as well as in a wide range of medicines, it is of primary importance to define its possible mutagenicity on human germ cells (ROEHRBORN, 1965). The various results of experiments with totally different test organisms will be discussed in detail later. We will begin with a short description of the distribution and the metabolism of caffeine in the human body.

Caffeine, regardless of its manner of application, distributes itself rapidly and evenly throughout the entire organism since it is highly water-soluble. The caffeine concentration in various tissues is directly correlated to their water content. An aqua-liquor barrier does not exist (AXELROD and REICHENTHAL, 1953). Caffeine can be transferred to an infant via its mother's milk.

The metabolism of caffeine occurs by way of demethylation and oxidation. There is no increase of uric acid in the urine; merely the excretion of monomethyl uric acid. Therefore, the authors thought that the demethylation is not complete; it progresses only up to monomethyl derivatives.

In the human system the metabolic rate for the methyl groups varies for the different positions: first, methyl is released from position N_3, then from position N_7 and rarely from the N_1-atom (CORNISH and CHRISTMAN, 1957). In other mammals, the methyl group is metabolized with a different position preference (FLASCHENTRAEGER and LEHNARTZ, 1954).

The elimination of caffeine from the body is relatively rapid. AXELROD and REICHENTHAL set the metabolic half-time for the human being at 3.5 hours; for a dog at 5 hours. Thus, a concentration of the substance over several days need not be considered. Even with a heavy coffee consumption, the body will eliminate the caffeine overnight. Test persons who drank 8 cups of coffee within 7 hours showed a total caffeine content of 180 mg (concentration in the serum times the water content in the body) in their body. This value is in the range of a therapeutic dose of 100–300 mg. The germ cells and even the foetus in the uterus of pregnant women are affected by the caffeine. GOLDSTEIN and WARREN (1962) proved this directly with operation candidates. Shortly before the operation they injected 1 mg caffeine sodium benzoate. The caffeine concentration in the plasma and the organs

1 Translated by S. KURTH.

2 The investigations of the author were supported by the "Deutsche Forschungsgemeinschaft".

(ovaries, testes, and foetuses) of the test persons proved to be virtually identical. Even the placenta is no protection against the caffeine. The authors estimate that the gonads of coffee drinkers are influenced by a constant caffeine concentration of 1 μg/ml.

Does this caffeine concentration have a mutagenic effect on the germ cells? Is there any caffeine mutagenicity on human beings? If so, in what manner does it work? The biologists of the last three decades have tried to answer these questions without any final solutions. One thing, however, is certain: the mutagenicity of caffeine on the human being is very weak, if any at all. For this reason, the problem of the experimental examination of weak mutagens will be treated in greater detail in the final discussion. A survey of the literature dealing with caffeine mutagenesis will precede this. We will not proceed chronologically, but organize the material according to the test organisms used. We will also consider what the results imply for the human being. Caffeine is an useful example to show the difficulty of applying the results of research with lower organisms and cell cultures to human beings. It shows once more how carefully one must be with either positive or negative statements.

A Survey of the Relevant Literature

Mutagenicity Tests with Bacteria

With bacteria the results of totally different mutagenicity tests with caffeine and other oxypurines have generally been positive. DEMEREC, BERTANI and FLINT (1951) found back mutations in Escherichia coli (streptomycin dependence to streptomycin independence). The maximum was reached with a caffeine concentration of 0.6% and a treatment duration of 48 hours. The mutation rate was more than four times that of the controls, i.e. from $7.9/10^8$ to $34.5/10^8$.

GEZELIUS and FRIES (1952) and NOVICK and SZILARD (1951) achieved similar results with the system of back mutations to phage resistance. The last named authors found the first indication of the mechanism of caffeine mutation by discovering that both the spontaneous and the oxypurine-induced mutations were dependent upon the growth limiting factor in the medium of the bacteria culture. Thus there is a connection between the spontaneous and the caffeine-induced mutations in terms of their manner of occurrence.

The research of KUBITSCHEK and BENDIGKEIT (1958–1964) lead to the following conclusions: The mutagenic effect of caffeine occurs on two levels: 1. It affects the DNA directly in cultures which are controlled by tryptophan in the medium. In these cases the increased mutation rate is independent of the number of generations which follow the treatment. 2. Cultures with other growth limiting factors show a mutation rate that depends on the number of generations following the treatment. This increase reaches its maximum at 3.4 generations. These two differing results indicate that there are, depending on the growth limiting factor, two ways in which caffeine can have a mutagenic effect: one that functions during DNA-synthesis and one that is independent of DNA-synthesis.

Speaking generally, there is an indirect influence of caffeine on replicating or non-replicating DNA because KOCH (1956), using labeled caffeine, proved that it is not directly incorporated into the DNA.

NOVICK's work (1956) discovered further characteristics which are common to both the caffeine-induced and spontaneous mutations. Both can be reduced or stopped through the natural purine nucleotides adenosine and guanosine. Evidently there exists an antagonistic effect between the oxypurines and the purine nucleotides. A further observation was that both the purine-induced and the spontaneous mutations showed a marked increase under aerobic conditions as opposed to anaerobic ones. Congruent with this observation is the fact that adenosine is found in bacteria only under anaerobe conditions. Adenosine thus causes a reduction in the mutation rate in anaerobe cultures. Unexpectedly, however, pyrimidin derivatives neither induced mutations nor did they, as nucleotides, display any antimutagenic activity.

The antagonistic effect of the caffeine and purine nucleotides is opposed by the synergistic effect of caffeine ad UV light which was examined by WITKIN (1958) and LIEB (1961). According to these examinations, an increase of up to 100% in the mutation rate of UV-light was achieved when caffeine was added to the culture immediately after the UV treatment. This result indicates that caffeine inhibits the repair process. Further research on this phenomenon by SAUERBIER (1964), METZGER (1964), DONESON and SHANKEL (1964), CLARKE (1967 and 1968), HARM (1967) and GRIGG (1968) has supported the theory that caffeine directly affects the enzymes of bacteria responsible for repair.

Mutagenicity Tests with Plants

Less informative in relation to the mechanism of activity but definitely positive are the results of mutation experiments with caffeine and other oxypurines (mainly 8-ethoxy caffeine) in plants. FRIES and KIHLMAN (1948) achieved a 10% increase of the mutation rate in ascomycetes Ophisotoma with a 2% caffeine solution and a treatment span of 5–8 days.

Cytological examinations of higher plants (Vicia faba, Allium cepa and Pisum sativum) lead to the following conclusions:

1. A 0.04% caffeine solution already causes a severe mitosis depression, which will reach 100% in 24 hours of treatment (KIHLMAN and LEVAN, 1949).

2. Slightly increased doses prevent the formation of the cell walls so that cells with several nuclei are formed. Fusion of these nuclei within a cell leads to the formation of gigantic nuclei with different degrees of polyploidy (GOSSELIN, 1940; MANGENOT and CARPENTER, 1944, 1946; KIHLMAN, 1949).

3. With the same low dose the chromosomes show so-called "primary effects" which can be seen in chromosome stickiness followed by the formation of pseudochiasmata (KIHLMAN, 1952b).

4. Higher doses of caffeine resulted in what KIHLMAN (1952a) called the "secondary effects". These are real chromosome aberrations. In anaphase chromosome bridges and fragment formation were observed. Because of their ressem-

blance to aberrations induced by X-rays, KIHLMAN speaks of a "radiomimetic effect" of the caffeine.

Different oxypurines proved to have various degrees of mutagenic effectivity. The degree of effectiveness depended upon the degree of methylation of the nitrogen atoms in the purine ring and the number of negative residues at the carbon atoms of the ring. An increase in the methylation and the introduction of negative groups increase the mutagenic effect whereas oxypurines with ethyl residues or longer carbon chains in place of the methyl groups were less effective.

On higher plants a synergistic effect of caffeine and oxygen has been described by KIHLMAN (1954 and 1955). Explanations for this phenomenon, to date, are purely hypothetical. This problem will recur in the discussion of our own experiments.

Experiments with labeled oxypurines enabled KIHLMAN's associates to prove that the mutagenic effect of the oxypurines is independent of DNA-synthesis in the higher plants and that they achieve their maximum effect in the G_2-phase of the cell cycle (KIHLMAN and ODMARK, 1965). It is assumed that the oxypurines change the physical properties of the DNA by attaching themselves to the DNA or by altering the conditions within the nucleus. This would lead to lesions during the re-organization into chromosomes in late interphase or early prophase. KIHLMAN and OVERGARD-HANSEN were unable to detect a direct influence on the various enzymes of DNA-synthesis.

Mutagenicity Tests with Drosophila

Various experiments of the mutagenic effects of caffeine on Drosophila had apparently contradictory results. ANDREW (1959) and OSTERTAG and HAAKE (1966) describe an increase of recessive X-chromosomal lethal mutations after caffeine treatment of drosophila males. Both, however, were unable to discover a specific, sensitive phase in the cycle of spermatogenesis of drosophila. OSTERTAG *et al.* as well as MITTLER, MITTLER and OWENS (1967) observed an increase in the X-chromosome loss after treatment of the female larvae. Contrary to MITTLER, MITTLER, TONETTI and SZYMCZAK (1967), OSTERTAG *et al.* found a reduction in the non-disjunction rate, but were unable to provide a statistical validation.

At the opposite end of the scale, there are the totally negative results of YANDERS and SEATON (1962) and ALDERSON and KHAN (1967). Neither found induced recessive X-chromosomal lethals in the males after caffeine treatment. They only found a slight increase in the non-disjunction rate, which they attribute to the stickiness effects which were already observed in plants. CLARK and CLARK (1968) also observed only an X-chromosome loss and a reduction in the non-disjunction rate and could find no X-chromosomal recessive lethal mutations. Thus it is with Drosophila that the first serious doubts as to the mutagenicity of caffeine appear. These contradictory results also indicate the importance of the choice of the test organism or system for the research in chemical mutagenicity.

BATEMAN (1969) points to the heterogeneity of the methods in the experiments of the authors mentioned so far and tried to compare them with his own data. The statistical significance of the results -- in increase or decrease of non-disjunc-

tion or chromosome loss—are primarily based on the large test material instead of on the magnitude of the effect, which is only two-fold that of the controls. He concludes that the criteria for the induction of non-disjunction or chromosome loss in Drosophila are not very suitable for expressing a genetic effect of caffeine.

Mutagenicity Tests with Human Cells *in vitro*

With human cell cultures, on the other hand, all the mutation experiments again showed positive results. As early as 1959, FRACCARO and NYMAN described a reduction of the mitotic rate in cultures of fetal human kidney specimens after 8-aethoxycaffeine treatment. Chromosome loss, acentric fragments, chromatid breaks and isochromatid breaks were found by JACKSON (1964) in leukocyte cultures which had been treated with 8-ethoxycaffeine. With a concentration of 4 mM/l for a 12 hour period followed by a 14 hour recovery time, the breakage rate increased, altogether, from 4–22%.

Further experiments with leukocyte and HeLa-cell cultures were carried out by OSTERTAG, DUISBERG and STUERMANN (1965). The results were as follows:

1. With dose of 500–10,000 μg caffeine/ml medium and a treatment duration of 1 hour followed by a 24 hour recovery period the breakage increased. At first the increase was linear to the dose. With the higher doses, however, the increase was more than linear (Table 1).

Table 1. *Number of breaks induced by caffeine per chromosome* (OSTERTAG *et al.*, 1965). *Chromosome analysis 24 hr after 1-hr exposure*

Caffeine (μg/ml)	Number of chromosomes	Chromatid + isolocus breaks		Trans-locations	Dicentrics	Rings
		number	per chromosome			
0	4,187	4	0.0009	—	—	—
500	2,754	18	0.0065	—	1	—
1,000	3,693	47	0.0128	*	2	—
2,000	4,361	111	0.0250	1	—	—
3,000	2,663	106	0.0398	1	2	—
5,000	5,666	401	0.0708	5	2	3
10,000	3,456	763	0.221	11	3	—

* 2 cells with 10 translocations.

2. Reunion figures were very rare.

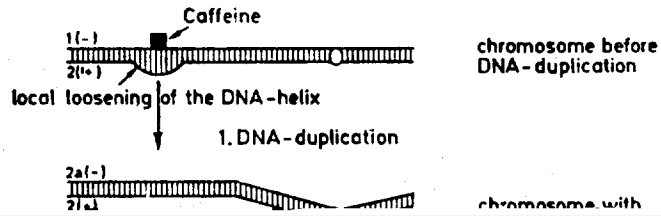
3. By varying the interim between the treatment and the fixation of the cells the earliest point at which chromatid breaks were observed was 5 hours after treatment. The maximum lay between 18 and 28 hours. After 20 hours, the first chromosome breaks appeared and after 45 hours there were only chromosome breaks left.

From these facts, OSTERTAG (1966) drew the following conclusions:

In relation to 1.: The dose/effect curve continues its linear course with doses below the tested 500 μg/ml concentration.

In relation to 2.: The break surfaces of the caffeine induced breaks have lost the ability to re-fuse.

In relation to 3.: The maximum of break induction is found in the middle to late G_1-phase of the cell-cycle and the DNA-synthesis phase is necessary to make the induced lesions visible. The G_2-phase is relatively insensitive to caffeine.



ting during the following S-phase. The other strand of the DNA replicates normally. Thus, after the first DNA-synthesis, a chromatid break appears. A further replication could then result in a chromosome break (Fig. 1).

OSTERTAG and GREIF (1967) showed, moreover, that the breaks are not equally distributed among the chromosomes. According to the phase of the cell-cycle in which the treatment occurred, different chromosome groups showed different breakage frequencies. In the G_1-phase, the A-group chromosomes showed a greater number of breaks than an equal distribution over the entire genome would permit. The B- and C-chromosome breaks corresponded to the expected values but the break frequencies of the D-, E-, and F-chromosomes were below the expected values. On the other hand, if the authors treated the culture during the S-phase, the B- and A_3-chromosomes showed an increase as compared to the expected values whereas the C-chromosomes showed less. Altogether, the experiments showed that the longer DNA-synthesis has progressed at the time of treatment the fewer caffeine-induced breaks there are. The breaks that do occur then, primarily involve the late replicating chromosomes. Independent of the phases, breakage occurred with a distinct preference for the telomer region of all chromosomes.

Mutagenicity Experiments with Mammals *in vivo*

After the above described results, there arises the urgent demand to test the mutagenic effects of caffeine on organisms which are physiologically closer to the human being and which permit *in vivo* experimentation. Such research was undertaken as early as 1928–1931 by STIEVE. STIEVE gave Russian rabbits daily subcutaneous injections of caffeine in doses of 0.05–0.2 g/kg body weight. Daily injections of 0.2 g/kg caffeine were reached via a weekly increase in the doses of 0.025 g/kg. The rabbits could tolerate such a high dose because their system had been habituated to the caffeine through the previous low doses. Isolated injections of 0.2 g/kg usually proved to be lethal. The histological examination of the testes and ovaries of thus treated male and female rabbits showed no evidence of change. Only the animals whose entire constitution was severely affected (with cramps, paralysis, loss of fur and weight) showed changes in their gonads: the testes tubuli still had normal germinal epithelium but there were no mature spermatids in the lumen; the ovaries showed only primary follicles. The larger follicles were all in various stages of regression. These symptoms, however, are only indirectly related to the caffeine.

Matings between treated males and normal females showed no decrease in the litter size in comparison to those of the control matings. The mating of animals before treatment served as the control. However, up to 70% of the litters did not survive the first week after birth. From this fact, one could deduce the induction of dominant lethals, which have been defined as "causing death before attaining the procreative age" (HADORN, 1949). Nowadays, however, based on the results of mutation research with X-rays and chemicals, it is generally accepted that induced lethal mutations already cause the intra-uterine death of the foetus. This was not the case in STIEVE's experiments with pure

caffeine and coffee containing caffeine (STIEVE, 1931). Other considerations as well indicate that outside circumstances influenced the death of the young during the first week. 1. The death of the young rabbits was limited to specific months; there could easily have been some sort of infectious disease. 2. In most cases, either the entire litter or none at all died. This indicates external causes since lethal mutations would have been equally distributed among the litters. 3. The animals were not kept in air-conditioned rooms so that weather conditions cannot be ignored as a possible detrimental factor. 4. The age of the males at the time of the control matings differs considerably from that at the time of the experimental matings. There was no control group running simultaneously with the experiments. Moreover, since the control material is very small ($\sim$ 1/5 of the experiment) the survival of the control group litters might have been accidental.

The experiments with the female rabbits showed only a reversible physiological effect on the germ cells, in that the animals were temporarily sterile.

The treatment of pregnant rabbits showed that the caffeine had a slight teratogenic effect. 32% of the pregnant females gave birth to dead litters. On the other hand, the other 68% had entirely normal litters of 5.3 young/female of which 72.4% survived the first week after birth.

Experiments done by EICHLER and MUEGGE (1931), using male and female rats which were treated before and during mating with 0.05 and 0.7 g/kg caffeine body weight for four generations showed no sign at all of either mutagenic effects or teratogenic ones.

The first mutagenicity experiments with mammals in a stricter sense were published as late as 1962. LYON, PHILLIPS and SEARLE treated C3H/HeH females and 101/H males for a duration of 7 weeks with 0.1% caffeine in the drinking water. The offsprings of these matings were then treated for 10 weeks. It was calculated that each F_1 animal had consumed a total of 0.3g caffeine. After this, the F_1 animals were mated with PCT partners who carried homozygous recessive genes in seven loci. The F_2 generation was then examined as to dominant visible mutations or recessive mutations in any of the seven loci. The results were absolutely negative (Table 2).

Table 2. *Specific locus and dominant visible (LYON et al., 1962) mutations following caffeine treatment*

Offspring of	Total progeny observed	Specific locus mutants	Mutation-rate/ locus/gamete $\times 10^5$	95% fiducial limits $\times 10^5$	Dominant visibles
Treated males	32,463	1	0.44	0.01–2.45	1
Treated females	31,686	0	0	0.00–1.66	1

In a further experiment, treated males were mated in a 1:5 ratio four weeks after the cessation of the caffeine treatment. The pregnant females were examined for dead implantations and corpora lutea graviditatis. The examination provided no evidence of caffeine-induced lethal mutations (Table 3).

At the same time, CATTANACH (1962) treated JU inbred males for three months with 0.3% caffeine in the drinking water. After this, he mated them in

Table 3. *Frequency of embryonic loss following outcrosses of caffeine treated males* (LYON *et al.*, 1962)

Caffeine concentration (%)	No. of males	Corpora lutea	Total implants	Live implants	Pre-implantation loss (%)	Implant loss (%)
0.05	2	264	200	187	24.2 ± 2.6	6.5 ± 1.7
0.1	6	743	577	522	22.3 ± 1.5	9.5 ± 1.2
0.2	7	682	522	468	22.5 ± 1.6	10.3 ± 1.3
Control	8	2,619	2,037	1,836	22.2 ± 0.8	9.9 ± 0.7

a 1:3 ratio over six weeks, replacing fertilized females with virgin females. The litters born in the first three weeks after the treatment were slightly smaller than those of the controls, but increased again as the time gap increased. In the first three weeks after treatment a relatively high number of sterile matings were observed. The F_1 animals showed no visible dominant mutations. The males were examined for evidence of possible reciprocal translocations. All F_1 males with litters numbering less than 8 were considered translocation suspects. In accordance with this criterium, the F_1 males were mated at the age of six weeks with Q-females in a 1:2 ratio. Of the 201 males so tested, there were only 13 animals whose litters numbered less than 8. The testes of these animals were cytogenetically examined in order to find any extant translocations. The criterium for supposing a translocation was a minimum of 8 meiotic metaphases with 19 instead of 20 bivalents. Only 2 of the 13 males provided "cytologically abnormal pictures" but these gave no clear evidence of a translocation. Therefore, these experiments again provided no evidence of caffeine induced mutations. The few effects such as temporary sterility and decreased litter size in the first few weeks after treatment, are probably of a physiological nature. CATTANACH himself considered oligospermia (as a result of a reduction in the division rate of the germ cells) a more likely cause of the decreased litter size than induced lethal mutations.

The results of the experiments with mice were relatively unsatisfactory and in need of more extended study. Therefore, our Institute organized further experiments in order to find out the effect of caffeine on mice.

Male mice of the C3H inbred strain were treated, at the age of ten weeks, either with a single dose of 0.2 g caffeine/kg body weight or with a daily dose of 0.25 g caffeine/kg body weight for three weeks. After treatment, the testes of these animals were prepared for the analysis of the first meiotic division according to the method of EVANS, BRECKON and FORD (1964) (modified by SCHLEIERMACHER, 1966). The interim between treatment and preparation in the 0.2 g treatment group was chosen according to OAKBERG's (1957) time table of spermatogenesis. This permitted the fractionated examination of various prophase stages of meiosis. The daily treatment during 3 weeks served for the examination of the entire premeiotic spermatogenesis, including the last DNA-synthesis phase before meiosis. Translocation, breaks and supernumerary bivalents were rare, less than 1% for both the treated and the control animals. Univalent chromosomes were more frequent but again there was no significant difference

Table 4. *Cytogenetic analysis of metaphase I (MI) figures in C3H inbred mice; 1. after a single treatment (0.20 g/kg); 2. after daily treatment for three weeks (0.25 g/kg)*

Treated stage of spermatogenesis	Number of		Percent univalent chromosomes		Total % of univalents
	mice	chromosomes	XY	autosomes	
1. Diplotene	2	4,000	0.30	0.25	0.55
Control	2	4,000	0.15	0.20	0.35
Pachytene	2	4,000	0.30	0.10	0.40
Control	2	4,000	0.45	0.30	0.75
Leptotene	1	4,000	0.30	0.25	0.55
Control	1	4,000	0.25	0.10	0.35
Resting spermatocytes	2	4,000	0.15	0.20	0.35
Control	2	4,000	0.15	0.20	0.35
All stages	7	16,000	0.26	0.20	0.46
Sum of control	7	16,000	0.25	0.20	0.45
2. Treated group	9	12,000	0.15	0.30	0.45
Control	3	12,000	$0.3\frac{-}{16}$	$0.1\frac{-}{16}$	$0.4\frac{-}{3}$

between the experimental animals and the controls (Table 4) (ADLER, 1966 and 1968; ADLER and RÖHRBORN, 1969).

In order to test for induced dominant lethal mutations and to establish specific sensitive phases in the spermatogenesis of mice, C3H, ten-week old males were treated with a single dose of 0.25 g caffeine/kg body weight. After treatment, they were mated with 3 virgin C3H females weekly. The different stages of spermatogenesis were individually covered during eight weekly breedings. The pregnant females were examined for dead implantations and corpora lutea graviditatis, 14.5 to 16.5 days post conceptionem. This extensive experiment again provided no evidence pointing to caffeine induced dominant lethal mutations, either in any single one of the tested phases of spermatogenesis or in the entire spermatogenesis (ADLER, 1969) (Table 5). Comparable experiments done by EPSTEIN (1968) also had negative results.

A cytological analysis of mitosis after a single *in vivo* treatment with 0.25 g caffeine/kg body weight of S_2-sarcoma cells in white mice of the AB/Jena Gat. strain showed no increase in the aberration rate over that of the controls. On the other hand, a combined treatment of caffeine and oxygen clearly influenced the frequency of breakage (Table 6). Like OSTERTAG'S, these were mostly open chromatid breaks (ADLER and SCHOENEICH, 1967). We will deal with the interpretation of this phenomenon in the later discussion.

Contrary to these results, KUHLMANN, FROMME, HEEGE and OSTERTAG (1968) reported the induction of dominant lethal mutations after chronic caffeine treatment in the drinking water of C57Bl males. They began treating the animals at the age of 2 months and continued the treatment for several months with doses of 0.025–0.85%. After an interim treatment with tap water in order to eliminate the physiological influences, these animals were mated with untreated C3H females. After the higher caffeine doses, the experimental animals showed an

Table 5. *Pre- and postimplantation egg loss after caffeine treatment of male C3H inbred mice (0.25 g/kg)*

Series (stage)	Treated ♂♂		Fertile ♀♀		No. of lutea corpora		No. of Implants		% PRE		% DI	
	A	B	A	B	A	B	A	B	A	B	A	B
I (sperm)	29	10	29	28	294	285	255	243	13.3	14.7	14.5	14.8
II (spermatids)	29	10	43	26	468	275	391	234	16.5	14.6	13.6	11.1
III (spermatids)	29	10	52	27	543	289	479	248	11.8	14.2	15.7	11.7
IV (spermatocytes)	29	10	49	23	517	252	464	226	10.3	10.3	13.8	11.9
V (spermatocytes)	29	10	55	23	589	235	527	197	10.5	16.2	15.2	17.3
VI (spermatogonia)	29	10	48	25	523	284	438	251	16.3	11.6	19.2	16.3
VII (spermatogonia)	29	10	49	22	511	229	463	198	9.4	13.5	13.2	15.2
VIII (spermatogonia)	29	10	48	27	531	291	478	246	10.0	15.5	14.6	11.0
Σ	29	10	373	201	3,976	2,139	3,495	1,843	12.1	13.8	15.0	13.6

A = treated group; B = control.

Table 6. *Chromosome-aberrations in cells of the S_2-sarcoma of mice after treatment with caffeine and combined treatment of caffeine and oxygen* (ADLER and SCHÖNEICH, 1967)

Treatment	Number of animals	Number of cells scored	Number of metaphases with					% cells with aberrations
			chromatid		reunion		rings	
			breaks	(%)	figures	(%)		
Caffeine	7	1,150	9	(0.8)	1	(0.09)	1	0.96
Control Aqua dest.	2	1,000	6	(0.6)	2	(0.2)	—	0.8
Caffeine and O_2	4	1,000	44	(4.4)	5	(0.5)	1	5.0
Control O_2	2	1,000	10	(1)	8	(0.8)	—	1.8

increase in the rate of dead implantations and in the pre-implantation egg loss compared to the controls. Unfortunately, the contol group was very small and did not run strictly parallel to the experiment (Table 7). The authors have postulated a dose/effect curve on the basis of this pre- and postimplantation egg loss, which is linear for the lower doses but levels off for the higher ones.

Since these experiments permit contradictory interpretations (see the discussion), further, more extensive experiments with chronic caffeine treatment were undertaken in our Institute (ROEHRBORN, 1969). The experiments were so planned that physiological and non-physiological high doses could be examined

Table 7. (KUHLMANN *et al.*, 1968)

Caffeine concentration[a] (%)	Matings	Pregnant animals	Corpora lutea[b]	Implantation	Living embryos	Preimplantation losses		Implant losses		Total loss (%)
						No.	%[c]	No.	%[d]	
0.025	10	8	54	41	35	13	24	6	14	35
0.05	11	10	82	55	44	27	34	11	20	46
0.1	16	14	120	88	74	32	27	14	16	38
0.2	15	8	68	36	29	32	47	7	19	57
0.3	16	11	83	56	36	27	33	20	36	57
0.5	13	10	74	41	27	33	43	14	34	64
0.025–0.5	81	61	481	317	245	164	34	72	22	49
Contr.	11	8	64	52	50	12	19	2	4	22

Dominant lethals induced by caffeine in mice.
[a] Treated for 100 days; 0.2 and 0.4% for 140 days.
[b] When the Corpora lutea could not be counted unambiguously, the average number 8 (377:47) has been used.
[c] % of total number of Corpora lutea.
[d] % of total number of implantations.

side by side in order to establish possible threshold effects. ROEHRBORN treated C3H males and $(101 \times C3H)F_1$ males, beginning at the age of 3 weeks, with doses between 0.0023% and 0.5% caffeine in the drinking water. After varying periods of treatment (up to 351 days) he mated the animals with 10–12 week old females of the corresponding strain. The 0.0023% dose corresponds to a human consumption of 2 cups of coffee. The fourfold dose of 0.01% was chosen

Table 8. *Experimental results with the dominant lethal method after chronic application of 3 caffeine doses to C3H-♂♂* (RÖHRBORN, 1969)

Experiment	Duration of treatment	No. corpora lutea	No. implants	% PRE[a]	No. DI[b]	% DI[c]	Probability	% PRE + DI4[d]	Probability
Caffeine 0.0023%	245	877	802	8.6	99	12.3	~0.36	19.8	~0.66
tapwater (control)	245	242	219	9.5	22	10.0		18.6	
Caffeine 0.01%	91	2,163	1,911	11.7	268	14.0	~0.015	24.0	~0.03
tapwater (control)	91	649	577	11.1	58	10.1		20.0	
Caffeine 0.01%	245	679	603	11.2	76	12.6	~0.20	22.4	~0.07
tapwater (control)	245	130	120	7.7	10	8.3		15.4	
Caffeine 0.5%	146	820	741	9.6	136	18.4	~0.68	26.2	~0.44
tapwater (control)	146	335	307	8.4	53	17.3		24.2	
Caffeine 0.5%	351	1,460	1,292	11.5	177	13.7	~0.90	22.1	~0.2
tapwater (control)	351	796	727	8.7	101	13.9		21.4	

$\chi^2 = 8.92$. $P^{[5]} \sim 0.10$. $\chi^2 = 9.93$, $P^{[5]} \sim 0.08$.
[a] Preimplantation egg losses in percent of the numbers of corpora lutea graviditatis.
[b] DI = dead implants.
[c] In % of the total number of implants.
[d] In % of the number of corpora lutea graviditatis.

Table 9. *Experimental results with the dominant lethal method after chronic oral application of low caffeine doses to (101 × C3H)-F_1-♂♂*[a] (RÖHRBORN, 1969)

Experiment	Duration of treatment in days	% dead implants[b]	Probability	% pre-implantation egg loss[c]	% pre- and post-implantation egg loss[c]	Probability
Caffeine 0.0023%	245	6.9	0.8	12.5	18.6	0.2
tapwater (control)	245	7.1		9.9	16.3	
Caffeine 0.01%	105	7.7	0.7	11.2	18.0	0.4
tapwater (control)	105	8.6		7.2	15.2	
Caffeine 0.01%	245	7.6	0.5	8.8	15.8	0.9
tapwater (control)	245	7.0		9.7	16.0	

[a] At the beginning of the experiments all ♂♂ had been 3 weeks of age; after the end of the treatment the ♂♂ had been mated with 10–12 weeks old (101 × C3H)-F_1-♀♀.
[b] In % of the total number of implants.
[c] In % of the number of corpora lutea.

in order to compensate the comparatively higher metabolic rate in the mice, whereas the third, non-physiological dose of 0.5% was chosen in order to cover any and all possible effects. The treatment of C3H males with the various doses showed, after 91 days, a weakly significant increase in the rate of dead implantations over controls only for the middle dosage group (Table 8). A second mating of the animals after 246 days of treatment did not reconfirm the former results so that an accidental increase in the first mating was assumed. The other dosage groups showed no differences to control values, either in the rate of dead implantations or in the pre-implantation egg loss. Examinations of the (101 × C3H)F_1 hybrids with doses of 0.0023% and 0.01% had only negative results (Table 9).

A third experimental group of 6 week old C57Bl males, kindly provided by KUHLMANN, received only the high dose of 0.5% caffeine in the drinking water and were mated after 161 and 245 days of treatment, with (101 × C3H)F_1 females. The first mating showed a statistically significant increase of the pre-implantation egg loss which, however, was not substantiated in the second mating (Table 10). It is unclear whether the difference between the first and the second matings is to be attributed to numerically insufficient material or to an increased tolerance of the caffeine. The discussion will also take a closer look at the meaning of the pre-implantation egg loss. Let it be said, however, that the post-implantation egg loss, i.e. the dead implantations, can be taken as the more conclusive criterium for induced dominant lethal mutations. These values did not vary significantly from those of the controls.

The treatment of (101 × C3H)F_1 females with a single injection of 0.25 g caffeine/kg body weight has, to date, shown no indication of induced dominant lethals (Table 11).

The animals of all groups in these extensive experiments will continue to receive treatment and will be mated again at a later date.

Table 10. *Experimental results with the dominant lethal method after chronic oral treatment of C57 BL males*[a] *with an extremely high caffeine concentration* (RÖHRBORN, 1969)

Drinking water	Days of treatment	No. fertile ♀♀	No. corpora lutea	No. implants	No. implants per ♀	No. living embryos	No. living embryos per ♀	% PRE[b]	Probability	No. DI[c]	% DI[d]	Probability
0.5% caffeine	161	62	607	465	7.50	428	6.90	23.4	$P \sim 0.0027$	37	8.0	$P \sim 0.8$
tapwater (control)	161	15	148	130	8.67	119	7.93	12.2		11	8.5	
0.5% caffeine	245	48	444	375	7.81	350	7.29	15.5	$P \sim 0.26$	25	6.7	$P \sim 0.74$
tapwater (control)	245	10	99	88	8.80	83	8.30	11.1		5	5.7	

[a] Outcrossed with 10–12 weeks old (101 × C3H)F_1♀♀.
[b] PRE = preimplantation egg loss.
[c] DI = dead implants.
[d] In % of the total number of implants.

Table 11. *Preliminary experimental results with the dominant lethal method after a single intraperitoneal injection of (101 × C3H)F_1-♀♀*[a] *with 0.25 g/kg caffeine* (RÖHRBORN, 1969)

Matings post-injection (days)	No. fertilized ♀♀		No. corpora lutea		No. implants		% preimpl. egg loss		No. dead implants		% dead implants	
	E[b]	CO	E	CO	E	CO	E	CO	E	CO	E	CO
0– 1	8	5	80	55	74	49	7.5	10.9	7	3	9.5	6.1
2– 8	21	9	197	85	173	80	12.2	5.9	18	4	10.4	5.0
9–15	33	10	302	97	274	89	9.3	8.2	27	12	9.9	13.5
16–22	36	10	335	107	294	100	12.2	6.5	26	7	8.8	7.0
23–29	39	9	391	90	351	79	10.2	12.2	37	5	10.5	6.3
30–36	14	9	142	96	124	86	12.7	10.4	6	13	4.8	15.1
Σ 0–36	151	52	1,447	530	1,290	483	10.9	8.9	121	44	9.4	9.1

[a] Age at the time of treatment: 10 weeks ± 2 days. Age of the (101 × C3H)F_1-♂♂ at the time of mating: 10–11 weeks.
[b] E = caffeine series; CO = control.

From single groups of C3H and hybrid males, some animals were taken after 245–351 days in order to do a cytological examination of the first meiotic division. The preparation method was the same as that used for the one-time treated animals. Even after chronic treatment, no increase of chromosome aberrations compared to the controls could be found (Table 12). The rates of univalent chromosomes were found to be within the range of the controls in most groups. Only one group (C3H males, 0.01%) showed a significant increase of the univalent autosomes. This result was regarded as accidental (ADLER and ROEHRBORN, 1969) since the results of groups treated with a considerably higher dose did not substantiate it.

Contr. 27 will describe the only statistical investigation concerning the direct effect of caffeine on a human population.

Table 12. *Cytogenetic analysis of metaphase I (M I) figures after chronical treatment of male mice with various caffeine doses* (ADLER and RÖHRBORN, 1969)

Strain	Treatment		Number of		Average daily caffeine intake in mg/kg body weight	Percent univalent chromosomes		Total % of univalents	Number of translocations
	caffeine concentration (%)	days	mice	chromosomes[a]		X-Y	autosomes		
C3H	0.0023	245	2	7,999	3.6	0.16	0.03	0.19	0
C3H	0	245	2	7,999	—	0.11	0.23	0.34	0
C3H	0.01	246	2	12,000	14.9	0.25	0.45	0.70	0
C3H	0	246	2	12,000	—	0.17	0.15	0.32	0[b]
C3H	0.5	351	1	8,000	304.9	0.25	0.15	0.40	0
C3H	0	351	1	8,000	—	0.10	0.15	0.25	1[c]
(101 × C3H)F_1	0.0023	245	2	8,000	3.4	0.15	0.15	0.30	0
(101 × C3H)F_1	0	245	3	8,000	—	0.20	0.15	0.35	0
(101 × C3H)F_1	0.01	274	2	16,000	14.9	0.13	0.08	0.21	0
(101 × C3H)F_1	0	274	2	8,000	—	0.20	0.15	0.35	0

[a] Each bivalent was counted as two chromosomes.
[b] Furthermore three questionable translocations which could not be clearly identified.
[c] One MI figure with one fragment.

Discussion

The suspicion, mentioned at the beginning of the paper, that caffeine might be incorporated into the DNA in the place of a purine base has been proved unfounded for bacteria by KOCH's (1956) experiments. In order to be incorporated, caffeine (for steric reasons) would have to be demethylated totally or at least in the position of N_1-atom. This is not the case in human beings (see Introduction). Therefore, it can be assumed that no incorporation of caffeine into the DNA (instead of a purine base) takes place in the human being.

Different conditions obviously apply in the cases of cells of higher plants or human cell cultures. In the latter OSTERTAG (1966) described a caffeine effect dependent upon DNA-synthesis and in the former KUHLMAN and ODMARK (1965) described an effect independent of DNA-synthesis. However, both authors do not assume a direct incorporation but merely a vague caffeine accumulation on the DNA.

The caffeine effect on the repair processes that was found by the bacteria geneticists was also found in mammalian cell cultures by some authors. RAUTH (1967) reported that caffeine inhibits the repair of UV-light induced lesions in 1-cell cultures of mice. Caffeine is also reported to increase the number of X-ray induced chromosome breaks in leukocyte cultures (JACOBSON, cit. KUHLMANN *et al.*, 1968). Finally, WRAGG, CARR and ROSS (1967) proved that an increase in the polymerase activity in leukocyte cultures, induced by Mitomycin-C, can be inhibited by caffeine. Since all these experiments involve *in vitro* systems, the results must be verified in a wide range of *in vivo* mammalian systems.

WOLFF (1968) found no indication that caffeine inhibits the repair process of X-ray induced chromosome breaks in Vicia faba. At present such a combination experiment with H_2O_2+caffeine and dimethyl sulfate+caffeine using ascites tumors of the mouse as the cytogenetic system showed negative results (SCHOENRICH, MICHAELIS and RIEGER).

Much more detailed research in respect to caffeine's influence on repair *in vivo* has been done by EPSTEIN, described in Contribution.

The synergistic effect of caffeine and oxygen in relation to break induction in Vicia faba (KUHLMANN, 1954) and S_2-sarcoma cells (ADLER and SCHOENEICH, 1967) is still unexplained. Based on KEILIN (1943) who showed that caffeine derivatives and haematins can form complexes, one could hypothesize that the effect is an inactivation of the peroxidase (which contains a haem-group) in the examined tissues. If oxygen forms peroxidases in a watery milieu and the peroxidases cannot be removed then a mutagenic effect of these peroxidases is possible. SCHOENEICH (1967) showed that hydrogen peroxide causes chromosome aberrations in S_2 sarcoma cells of mice.

This explanation is, however, only a speculation. KIHLMAN (1955b) indicated something of this nature but he has never mentioned it again.

The warning against caffeine as "one of the most dangerous mutagens in man" (OSTERTAG, 1966; KUHLMANN *et al.*, 1968) is based on calculations resulting from the experiments with cell cultures and depends upon the following suppositions:

1. the breakage frequency is directly proportional to the dose even in the doses below 500 μg/ml which were not tested.
2. Breakage occurs only after DNA replication.
3. The effect of caffeine is independent of the duration of treatment but dependent upon the number of cell generations.

Extremely complicated calculations of the mutagenic risk involved for the human being lead to the statement that normal coffee consumption of a human male causes a mutation risk of 12% for each germ cell.

Since women have no germ cell divisions between birth and maturation (ovulation of the egg cells) the risk is much smaller for them and involves only the embryonic development. Nonetheless, a 0.5% mutation risk is postulated.

Apart from the fact that these calculations are, at best, a very rough estimate (considering the number of human genes and the number of generations that a primitive germ cell must go through before it becomes a mature sperm or egg cell) there are some serious flaws in the reasoning. The calculations are based on break induction rates with 1,000 μg/ml in cell cultures. A possible threshold effect with smaller doses is simply neglected (OSTERTAG, 1966). The author himself qualifies his calculations in the following respects:

1. It is questionable whether one can equate chromosome breaks in cell cultures with actual mutations.
2. Caffeine-induced damage might be reparable.
3. Caffeine damaged sperm might be eliminated during the process of spermatogenesis.

Moreover, the physiological conditions within an *in vitro* system are hardly suited to such a direct mathematical comparison with the conditions in a complex

living organism with all its resistances. Given the present state of knowledge, such calculations and dramatic warnings seem, to us, definitely premature.

At present there are no clearcut results demonstrating the mutagenic effects of caffeine on mammals. The early experiments with rabbits and rats (STIEVE, 1928, 1931; EICHLER and MUEGGE, 1931), apart from a few ambiguous points, returned only negative results. The same applies to the experiments of LYON, PHILLIPS and SEARLE (1962). The fertility reduction, described by CATTANACH (1964), could be attributed to pre-implantation egg loss; in part, at least, it can be physiologically explained.

KUHLMANN'S experiments which showed an increase in the pre-implantation egg loss and those by ROEHRBORN on C57Bl mice which showed the same after the first matings, make it necessary to take a closer look at this criterium. According to BATEMAN (1958), about 3/4 of all zygotes carrying *more* than one lethal factor are never implanted in the first place, whereas zygotes carrying only one lethal remain capable of implantation, and die at a later date. Only the latter would therefore appear as dead implantations—our criterium for the induction of a dominant lethal mutation. The problem is that the death of a zygote before implantation can have many purely physiological reasons: the ovulation of immature and therefore non-viable egg cells; physiological damage to the spermatozoa which affects the mitochondria or other cell organisms; a local disturbance of the endometrium which makes implantation impossible. Some of these factors should be in evidence in the controls as well since they are independent of the treatment. In KUHLMANN'S experiments the control material is, unfortunately, so small that a purely accidental absence of an increase of the pre-implantation egg loss in the controls is a very real possibility. If the pre-implantation egg loss can actually be traced, at least in part, to induced dominant lethals, and according to BATEMAN'S criterium, more than one lethal per sperm, then there is no reason to suppose that a weak mutagen would release several mutations in some cells and none at all in others. It would be more reasonable to assume that the individual incidence per germ cell would also increase. Such was not the case in ROEHRBORN'S experiment with C57Bl mice. Thus, KUHLMANN'S positive results require confirmation in larger series so that the conclusions may be validated.

KUHLMANN'S control values, for instance, are unusually low: 4% dead implantations. But these values are based on only 8 pregnant females. In ROEHRBORN'S matings, the rate of spontaneous dead implantations is almost twice that in three times the material (35 pregnant females and 7.9% dead implantations). In our experience, the rate of dead implantations in the controls is largely dependent on the strain to which the females belong. The rate is considerably lower for hybrid females than for inbred ones. Consequently, a much higher control rate ($\sim$10%) is to be expected in a mating of C57Bl males to C3H females (see RÖHRBORN, 1968).

According to a personal communication from KUHLMANN, his experimental values also include the dead implantations of those females who had *only* dead embryos. To include these in the evaluation seems a doubtful procedure since the death of *all* embryos certainly need not be due to dominant lethal mutations. A much more likely cause are physiological disturbances in the maternal organism.

That there was no case of complete embryo mortality among the controls could easily be due to the limited number of controls. Apart from this, the controls did not run strictly parallel to the experiment which means that an infection might have caused the mortality phenomenon in the experimental group (ROEHRBORN, 1969).

This detailed discussion was designed to show the importance, even for positive results, of having sufficiently extensive experimental material and, especially, the absolute necessity of having a large control group, running exactly parallel to the experiment.

The numerically much larger experiments with both the single high dose treatment as well as the chronic treatment with different doses on C3H and $(101 \times C3H)F_1$ animals showed negative results in the lethal mutation tests and in the analyses of the first meiotic divisions (ROEHRBORN, 1969; ADLER and ROEHRBORN, 1969; ADLER, 1966 and 1969).

However, these negative results do not exclude the possibility that a weak mutagenic effect of the caffeine might have been overlooked. In testing the difference between the rates observed in two sample populations (treated mice and controls) two types of error can be made: first to postulate a difference between the true rates in the two populations which, in reality, does not exist; second, to assume an equality of the true rates in a case of only a slight but real difference (VOGEL, 1967). This second kind of error is the danger in our case. We, therefore, tried to establish the validity of the negative results using the method described by STROBEL and VOGEL (1958). It is presented here, using the values of the lethal mutations test on mice after one-time treatment with the high caffeine dose. If we denote:

P_0, P_1 = true rates of dead implantations in controls resp. experiment;

n_0, n_1 = total number of implantations in controls resp. experiment;

k_0, k_1 = number of dead implantations in controls resp. experiment and

$K = k_0 + k_1$, and $t = k_1/k_0$;

$\chi^2_{0.1} = 2.706$ = upper 10% point of the χ^2 distribution with 1 df., formula 8 of these authors givens an upper 95% confidence limit R_U for the ratio $R = P_1/P_0$ of the true rates of the dead implantations:

$$R_U = \frac{n_0}{n_1}\left\{t + \frac{(1+t)^2}{2}\,\frac{\chi^2_{0.1}}{K} + \sqrt{t(1+t)^2\,\frac{\chi^2_{0.1}}{K} + \frac{(1+t)^4}{4}\cdot\frac{\chi^2_{0.1}}{K^2}}\right\}$$

In our experiment this confidence limit R_U had the value 1.25, meaning that with a probability of 95% the true rate of dead implantations in the experiment is at most 1.25 times higher than the true control rate. In other words, an average increase of 25% in the total number of dead implantations under the conditions of our experiment can still be in agreement with our results. Thus, our conclusion is statistically sound.

In conclusion, it should be stated that, despite the positive results with lower organisms, higher plants, drosophila (some) and particularly with human cell cultures, neither a statistical analysis nor *in vivo* experiments with mammalian systems have shown any mutagenic effects which could be definitely attributed

to caffeine. A premature warning against a substance that is so widely used and has such medical importance could do a great deal of harm. Given the results of our experiments with mammals, it seems most inadvisable to term caffeine as "one of the most dangerous mutagens in man". Whether and to what degree synergistic effects with caffeine and other substances occur remains to be defined in experiments with mammals.

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Cytogenetic Investigation of Meiotic Chromosomes of Male Mice after Chronic Caffeine Treatment*

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Summary. Male C3H inbred mice and male (101 × C3H)F_1 hybrids were treated with various caffeine doses over a long period. After the end of treatment the animals were dissected and the metaphase I figures of the cycle of spermatogenesis were analysed. The experiments revealed no distinct evidence for the induction of chromosomal aberrations in the cycle of spermatogenesis caused by caffeine up to metaphase I.

Caffeine is known as a mutagen in lower organisms (RÖHRBORN, 1965). Because of the wide-spread use in medicine and the intake by man in form of coffee especially this compound should be investigated very carefully in mammals by means of different methods. A comprehensive study with the dominant lethal method after chronic caffeine treatment will be published later (RÖHRBORN, 1969). Here we will only deal with the cytogenetic analysis of metaphase I chromosomes after chronic treatment of male mice.

Materials and Methods

In our experiments we used male mice of the C3H strain and male (101 × C3H)F_1 hybrids. The application of caffeine was started at an age of 3 weeks. The test males received the various caffeine concentrations in their drinking water. The caffeine concentrations and the average caffeine intake per day can be seen in Table 1. The caffeine concentrations were chosen in such a way that the lowest dose of 0,0023%, would correspond to a daily intake of about two cups of coffee by man. Since mice metabolize caffeine possibly faster than man, an about four times higher concentration of 0,01%, was given. The third concentration, the extremely high dose of 0,5%, caffeine, was chosen in order to find out whether this drug induces chromosome aberrations in the cycle of spermatogenesis at all. At the end of treatment the test males and their parallel running controls were dissected and preparations of meiotic chromosomes (diakinesis and metaphase I) were made according to the method of EVANS et al. (1964) (modification according to SCHLIEBERMACHER, 1966).

Results

The results of our analysis we summarized in Table 1. In all caffeine series neither translocations nor chromosome breaks were observed. The only clear-cut evidence for a translocation was found in a control male (Fig. 1). Furthermore in this control male we found three questionable translocations which could not be clearly identified. In another control male we observed one metaphase I figure

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Table 1. *Cytogenetic analysis of metaphase I (MI) figures after chronical treatment of male mice with various caffeine doses*

Strain	Treatment		Number of		Average daily caffeine intake in mg/kg body weight	Percent univalent chromosomes		Total % of univalents	Number of translocations
	caffeine concentration (%)	days	mice	chromosomes[a]		X-Y	autosomes		
C3H	0.0023	245	2	7999	3.6	0.16	0.03	0.19	0
C3H	0	245	2	7999	—	0.11	0.23	0.34	0
C3H	0.01	246	2	12000	14.9	0.25	0.45	0.70	0
C3H	0	246	2	12000	–	0.17	0.15	0.32	0[b]
C3H	0.5	351	1	8000	304.9	0.25	0.15	0.40	0
C3H	0	351	1	8000	–	0.10	0.15	0.25	1[c]
(101 / C3H) F_1	0.0023	245	2	8000	3.4	0.15	0.15	0.30	0
(101 / C3H) F_1	0	245	3	8000	–	0.20	0.15	0.35	0
(101 / C3H) F_1	0.01	274	2	16000	14.9	0.13	0.08	0.21	0
(101 / C3H) F_1	0	274	2	8000	–	0.20	0.15	0.35	0

[a] Each bivalent was counted as two chromosomes.

[b] Furthermore three questionable translocations which could not be clearly identified.

[c] One MI figure with one fragment.

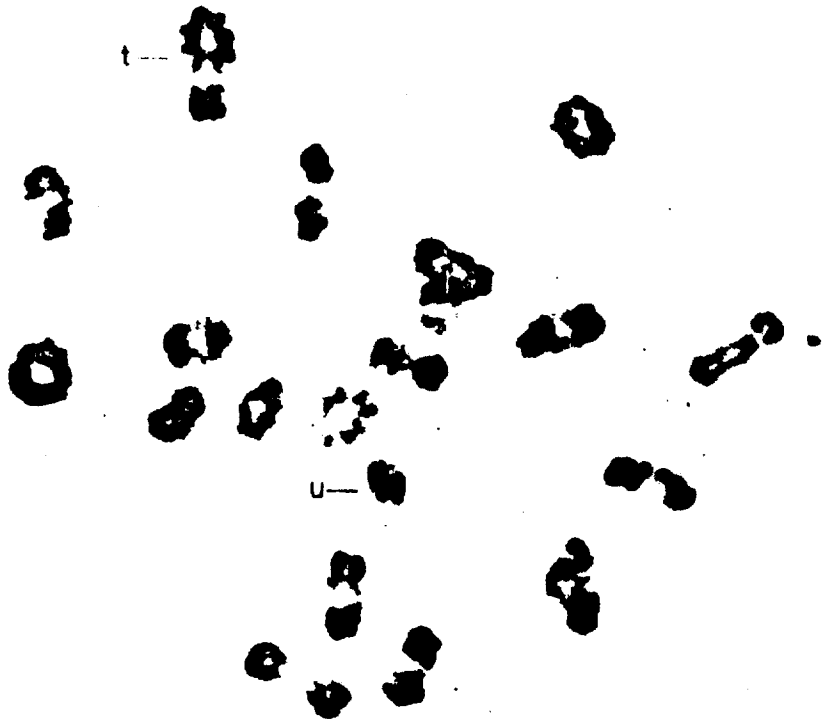

Fig 1. One trivalent (*t*) and one univalent (*u*) chromosome as the result of a reciprocal translocation

with a chromosome fragment. In the C3H strain the frequency of univalent chromosomes in both the 0.0023% and the 0.5% caffeine series was not significantly different from their controls. Only in the 0.01% series of C3H males we found a significant difference ($P = 0.0027$) between experiment and control with respect to the total frequency of univalent chromosomes. The significance was nearly exclusively due to the different ratios of the autosomal univalents ($P < 000027$). The problems concerned with these findings are discussed in the next Chapter. In the male (101 × C3H) F_1 hybrids the outcome was clearly negative. The ratios of gonosomal univalents in the caffeine series were even lower than in the corresponding controls. On the basis of these results we could exclude (using the method of STROBEL and VOGEL) that caffeine in hybrids causes an increase in the rates of univalent chromosomes as compared to the control (99% confidence level). Altogether we found 164 pairs of univalents in 2400 cells. In nearly all cases there was only one pair of univalents in one metaphase I. Only one cell of a control animal contained both one autosomal and one gonosomal univalent pair. Another cell of a control male contained two pairs of autosomal univalents such as one cell of the 0.01% C3H caffeine series. Therefore multiple occurrence of univalent chromosomes is quite rare and distributed uniformly in experiment and control. In one cell of the C3H 0.0023% caffeine series we found no X chromosome and in one cell of the corresponding control there was no Y chromo-

some. The uniqueness of these events and the occurrence in both control and experiment allows to assume that these both cases were only chance events or possibly an effect of preparation.

Discussion

Caffeine intake by man is usually more or less chronic. Therefore we treated the animals constantly over a long period. By such a treatment one might expect an accumulation of induced chromosomal aberrations in spermatogonia rather than induced aberrations in resting primary spermatocytes, leptotene, zygotene, pachytene, and diplotene. Most of these stages had been checked by ADLER in his thesis. The outcome was negative (see Table 2).

In our chronic experiments as mentioned above we found no structural chromosome aberrations in any of the caffeine series. Only in one of the five test series there was a significant increase in the frequency of univalent chromosomes (Table 1, 0.01% caffeine; C3H strain). In all other series the ratios of univalent chromosomes did not differ significantly from the corresponding controls. What does this mean? In metaphase I X and Y chromosomes are not correctly paired but associated end to end without observable chiasmata formation. Therefore, it is not surprising that XY univalents were found to be nearly as frequent as univalents of the 38 autosomes. Univalent autosomes in metaphase I meiosis can be the result of various causes. Thus the homologuous chromosomes could have been unpaired primarily by chance or because of heterozygous inversions and so on. On the other hand they might be the result of premature depairing. As a consequence of insufficient chiasma formation one, two or even four univalent chromosomes may also occur in a metaphase I figure in cases of heterozygous translocations. A good example are the univalent and trivalent chromosomes in the case of the heterozygous translocation shown in the figure. According to FORD and CLEGG (1969) the chiasma frequency in the male mouse is low and the "failure to form chiasmata in one or more arms of the pachytene

Table 2. *Cytogenetic analysis of metaphase I (MI) figures after a single treatment (0.20 g/l caffeine) of C3H males*

Treated stage of spermatogenesis	Number of mice	Number of chromosomes	Percent univalent chromosomes X-Y	Percent univalent chromosomes autosomes	Total % of univalent chromosomes
Diplotene	2	4000	0.30	0.25	0.55
Control	2	4000	0.15	0.20	0.35
Pachytene	2	4000	0.30	0.10	0.40
Control	2	4000	0.45	0.30	0.75
Leptotene	1	4000	0.30	0.25	0.55
Control	1	4000	0.25	0.10	0.35
Resting spermatocytes	2	4000	0.15	0.20	0.35
Control	2	4000	0.15	0.20	0.35
All stages	7	16000	0.26	0.20	0.46
All controls	7	16000	0.25	0.20	0.45

a common". Therefore we think that the occurrence of univalent chromosomes alone in metaphase I figures should not be over-estimated. Consequently significant difference between the ratio of univalent autosomes in experiment control of one of the C3H series we regard as chance event. Furthermore — mentioned before — the outcome of our hybrid series was clearly negative, for results are in good agreement with the findings of ADLER (1966) after fractionated administration of high caffeine doses to male mice. The nearly identical frequencies of univalent autosomes in the controls of C3H inbred males and 101 / C3H) F_1 hybrids indicate a structural similarity of the chromosomes of the strains 101 and C3H.

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Section of Endocrinology

President A Stuart Mason MD

Meeting April 28 1971

Cases

Metabolic Effects of Chronic Caffeine Administration in a Patient with Diabetes Mellitus

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Mr A P, aged 59

History: Admitted for investigation of hyperglycæmia. He had suffered from narcolepsy since adolescence with sharp deterioration in 1953 when he slept up to 20 hours per day. He was treated initially with dexamphetamine, then amphetamine sulphate, and more recently with caffeine up to 7 g/day, equivalent to fifty cups of instant coffee. In the eighteen months prior to admission in 1970 he lost 20 lb (9 kg) in weight and for six months had noticed deteriorating vision in his left eye. There was no family history of diabetes mellitus. On admission, as well as glycosuria, he had signs of weight loss and bilateral retinopathy with microaneurysms, hæmorrhages and exudates.

Investigations: Blood pressure normal. Hb 17·6 g/100 ml; blood cholesterol 140 mg/100 ml; blood glucose 325 mg/100 ml. Urine: 17-oxosteroids 5·5, 17-oxogenic steroids 21·1 mg 24 h. Intravenous glucose (IVGTT) and tolbutamide (IVTTT) tolerance tests were carried out on admission and again six days after caffeine therapy was stopped. Fasting serum insulin values were in the normal range but depressed for his concentration of blood glucose. Before caffeine was stopped the IVGTT (0·5 g/kg body weight) showed gross glucose intolerance with a biphasic, much reduced insulin response. The IVTTT showed a more normal insulin response, a peak of 42 microunits/ml being reached 5 min after tolbutamide. Fasting blood lactate and pyruvate levels were normal but showed no rise after i.v. glucose. After caffeine was stopped there was a small increase in peak insulin response after both glucose and tolbutamide but no change in glucose tolerance. Blood lactate and pyruvate also showed a moderate rise after glucose, rather than no change. The most dramatic changes concerned blood ketone bodies and free fatty acids (FFA) (Table 1). It can be seen that both 3-hydroxybutyrate and acetoacetate fell by over 50% after stopping caffeine therapy, while blood FFA showed a smaller fall. Blood triglycerides, however, showed no change.

Comment

Acute caffeine ingestion has been shown to diminish glucose tolerance and reduce pancreatic insulin release in response to glucose in normal subjects (Wachman *et al.* 1970) and maturity onset diabetics (Jankelson *et al.* 1967). It also increases FFA levels (Feinberg *et al.* 1968). In this man with a chronic high intake of caffeine no change in glucose tolerance and minimal changes in insulin secretion were noted when caffeine was withdrawn for six days. The major changes were a moderate fall in plasma FFA

Table 1
Fasting blood metabolite concentrations before and after withdrawal of caffeine

	On caffeine		*Off caffeine*	
3-hydroxybutyrate (mol/l)	0·361,	0·248	0·078,	0·104
Acetoacetate (mol/l)	0·175,	0·150	0·06[illegible],	0·071
Ratio 3-hydroxybutyrate/acetoacetate	2·12,	1·65	1·24,	1·49
FFA (mEq/l)	0·81,	0·81	0·75,	0·56
Triglycerides (mg/100 ml)	34,	44	47	52

Each value is the mean of two separate fasting samples

and a marked fall in circulating ketone bodies (Table 1). Caffeine may have increased adipose tissue cyclic 3',5'-AMP concentrations by inhibition of phosphodiesterase, thus stimulating lipolysis; there may also have been an increase in catecholamine release.

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THE INFLUENCE OF AGE ON THE EFFECTS OF CAFFEINE ON THE FUNCTION AND METABOLISM OF THE HEART

Dedicated to Professor Dr. F. Heim on his 60th Birthday

by

H.P.T. Ammon and C.-J. Estler

From the Pharmacological Institute of the University of Erlangen-Nuremberg; Director: Professor Dr. F.Heim

As is known, the effects of caffeine on the cardiocirculatory system consist above all in an increase in cardiac frequency, contractibility of the cardiac muscle, and a slight rise in blood pressure; thus, in an overall increase in cardiac effort. Closely connected with that are increased oxygen consumption and metabolism of energy-rich phosphoric acid esters in the cardiac muscle. Since today caffeine is consumed in large amounts by both young and old, and since age obviously has a certain influence on the effect of drugs, we intended, in an experiment with animals, to test whether quantitative differences between animals of various ages could be determined with respect to the effects of caffeine on the function of the heart and its energy-producing metabolism. Therefore, we examined, in rats aged from 15 to 18 months, the effect of caffeine on cardiac frequency and blood pressure and measured the total cardiac effort approximately from average blood pressure $\cdot \sqrt{\text{of the frequency}}$, the method of Bretschneider (1). Furthermore, the ATP and creatin phosphate content of the heart was determined, as a parameter for the cytoplasmic redoxstatus of the lactate/pyruvate quotient (2) and as a standard for the availability of chemical energy.

Methodology

We conducted our tests on Sprague Dawley rats, which were kept in large boxes at an ambient temperature of 24°C, provided with drinking water ad libitum and a standard diet of Altromin R* (Altromin GmbH, in Lage/Lippe). For the tests we used rats that had reached puberty, 2 month-old rats and 15-18 month-old rats. The animals were anesthetized with 1.2 g/kg urethane intraperitoneally, placed on a heated operating table and, after a tracheotomy, made to breathe artificially with a Starling respiration pump (Braun Co., Melsungen).

The old and young rats were divided into two groups; of these, one received 40 mg/kg caffeine intravenously, cal-

culated as a base in the form of caffeinum natrium benzoicum DAB 7, while the other (control group) received an equal amount of liquid cream 0.9% NaCl (0.1 ml per 100 g) intravenously.

The blood pressure was measured continuously 10 minutes before and 30 minutes after the injection of caffein or 0.9% NaCl; it was measured as an average pressure in the A. carotis with a Statham apparatus (Statham Miniature Blood Pressure Transducer Model SP 37) through the Hellige Program 19 pressure measuring device. At the same time, the cardiac frequency was determined with the Eka pulse device (Hugo Sachs Elektronik, Hugstetten) by means of electrodes situated subcutaneously. Blood pressure and cardiac frequency were registered on the 3-channel register of the firm of Hellige, in Freiburg.

Thirty minutes after administration of caffeine or 0.9% NaCl, the thorax was opened and the heart frozen between aluminum blocks cooled in liquid air. After adhering frozen blood was removed, the tissue was pulverized along with 4 parts of 3.5% frozen perchloric acid and then the thawing mixture was centrifuged at 0°C. In the thawed residue, we determined the adenosintriphosphate (ATP) and the creatin phosphate according to the method of Lamprecht and Stein (6), the pyruvate*) according to the method of Bücher (unpublished), and the lactate*) according to the method of Scholz, Schmitz, Bücher and Lampen (8).

The values given in figure 1 represent the deviations from the initial value after intake of caffeine or 0.9% NaCl. They are mean values from the number of tests indicated in a particular case and their average deviation (SEM). The values of figures 2 and 3 are likewise mean values and their average deviation (SEM), calculated according to the t-test of Student from the number of single tests indicated.

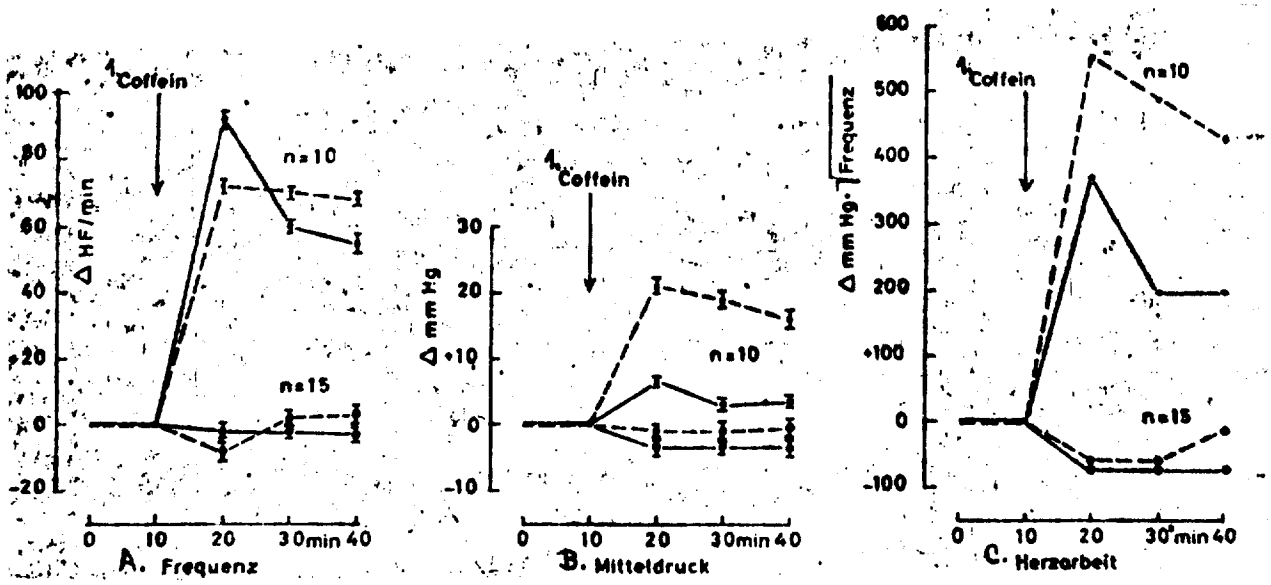

Fig. 1: Change in cardiac frequency, average blood pressure and the product of average blood pressure times the square root of the cardiac frequency in 2 month and 15-18 month-old

*) Biochemica Test Combination, Boehringer, Mannheim.

(fig. 1 cont'd) rats after intravenous introduction of 40 mg/kg caffeine and 0.1 ml/100 g 0.9% NaCl. Initial values: frequency, young: 322, old: 348. Average pressure: young: 65, old: 60 mm Hg.

———— 2 month-old rats · — — — 15-18 month-old rats
--•-- 40 mg/kg caffeine --o-- 0.9% NaCl, 0.1 ml/100 g

A. frequency B. average pressure C. cardiac effort
1. caffeine

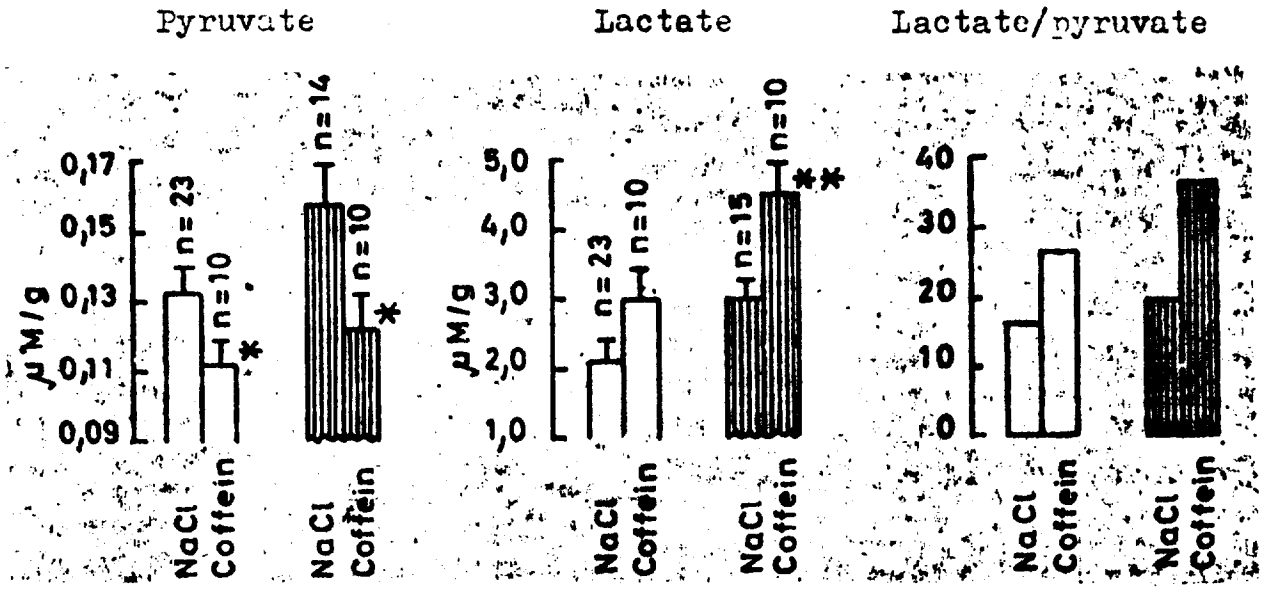

Fig. 2: Pyruvate and Lactate content of the heart, as well as lactate/pyruvate quotient, 30 min. after iv. administration of 40 mg/kg caffeine or 0.1 ml/100 g 0.9% NaCl to 2 month and 15-18 month-old rats. □ 2 months. ■ 15-18 months. *) $P<0.05$; **) $P<0.01$.

Coffein = caffeine

Results and Discussion

As fig. 1 shows, after intravenous introduction of caffeine, age-dependent differences in its effect on cardiac frequency and blood pressure are found. Ten minutes after administration of caffeine there is an increase in cardiac frequency in both the young and the older animals. While in the case of the young animals, the frequency drops again 20 minutes after caffeine administration, in the case of the older animals, it remains at about the same increased level during the entire experimental time of 30 minutes. In the case of the young animals, the blood pressure increases only slightly. This rise in pressure is moreover relatively temporary and lasts only 10 minutes. On the other hand, in the case of the older animals, it is significantly more marked

and also of longer duration. If we calculate the cardiac effort approximately from the product of average blood pressure times the square root of the frequency, then, as fig. 1 shows, it increases significantly more strongly in the case of the older animals than in the case of the younger.

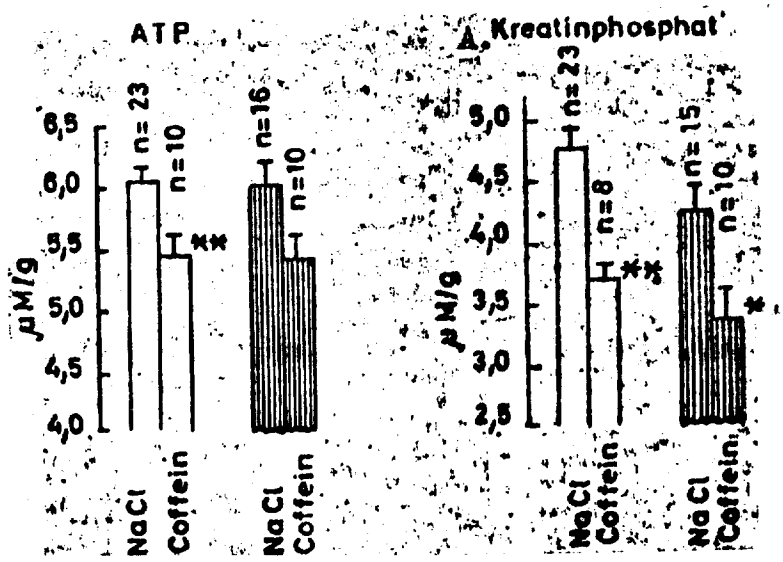

Fig. 3: Heart content in ATP and creatin phosphate 30 min after iv. administration of 40 mg/kg caffeine or 0.1 ml/100 g 0.9% NaCl to 2 month-old (□) and 15-18 month-old (■) rats. * $P < 0.05$; ** $P < 0.01$.
A. Creatin phosphate

Age-dependent differences are found after administration of caffeine in the cytoplasmic **redoxstatus** of the myocardium. As fig. 2 shows, in the young rats there is an increase in the lactate-pyruvate (l/p) quotients of 10.8. Even clearer is the increase in the older animals -- 17.0. As can be seen from fig. 2, the increase in the l/p quotient is based both on an increase in the pyruvate content and an increase in the lactate content in the heart. The increase in the l/p quotients that can be observed in both the young and older animals, and thus also that of the red./ox. quotient, is probably caused by the fact that, as a consequence of greater cardiac effort after caffeine intake, the oxygen collection from the myocardial tissue increases, since the increase in cardiac effort and oxygen consumption move parallel with one another (3). Since in the case of the older animals the effort achieved by the heart increases to a greater degree than in the case of the young animals, the greater increase in the l/p quotient in these animals could be a manifestation of a greater oxygen collection from the myocardium.

Coronary blood pervasion and intracellular **redoxstatus** are related to one another in that upon decrease is the oxygen pressure in the tissue, which sets in with an increase in the redox quotient, the filling of the myocardium with

blood increases.(7). Thus, the rise in the l/p quotients in the heart after administration of caffeine can be viewed in connection with an increase in coronary blood pervasion. This last will probably increase more in the older animals than in the young, due to the more marked rise in the l/p quotients.

However, in both groups, as can be seen from fig. 3, there is an nearly equal drop in ATP and creatin phosphate in the heart. These findings concur with the results obtained by other authors after administration of methylxanthine (5, 9), isoprenalin*) (4) and orciprenalin**) (Ammon, unpublished). Since all the substances named also lead to an increase in cardiac activity along with a decrease in ATP and creatin phosphate, it can be assumed that the decrease in energy-rich phosphoric acid esters is not based on a disturbed energy production, but rather that the content in energy-rich phosphates moves to a lower level within a physiological range.

Summary

After iv. administration of 40 mg/kg caffeine to anesthetized rats, age-dependent differences in the effect of caffeine on function and metabolism of the heart can be determined.

Cardiac frequency and especially blood pressure, as well as the cardiac activity calculated from the multiplication of average pressure with the square root of the rate increase more markedly in 15-18 month-old rats than 2 month-old rats.

The lactate/pyruvate (l/p) ratio of the heart increases by 17.0 in the older rats, and only by 10.8 in the young animals. The greater increase in the l/p ratio in the older animals is attributed, because of a greater increase in cardiac activity, on a greater extraction of oxygen from the tissue.

In contrast, there are no age-dependent differences in the effect of caffeine on the adenosintriphosphate and creatin phosphate content of the heart. Both decrease in the case of both the young and older animals, to the same degree.

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*) N*isopropyl-noradrenalin-sulfate.
**) 1-(3,5-dihydroxyphenyl)-2-isopropylaminoethanol-sulfate.

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Aus dem Pharmakologischen Institut der Universität Erlangen-Nürnberg; Direktor: Professor Dr. F. Heim

Der Einfluß des Alters bei den Wirkungen des Coffeins auf Funktion und Stoffwechsel des Herzens

Herrn Professor Dr. F. Heim zum 60. Geburtstag gewidmet

Von H. P. T. Ammon und C.-J. Estler

Die Wirkungen des Coffeins auf das Herz-Kreislauf-System bestehen, wie bekannt, in erster Linie in einer Steigerung der Herzfrequenz, der Kontraktilität des Herzmuskels und in einer leichten Erhöhung des Blutdruckes, also in einer Zunahme der Herzleistung. Damit eng verbunden sind erhöhter Sauerstoffverbrauch und Umsatz energiereicher Phosphorsäureester im Herzmuskel. Da Coffein heute in großem Umfang sowohl von jungen als auch von älteren Menschen aufgenommen wird und das Alter offenbar einen gewissen Einfluß auf die Wirkung von Pharmaka besitzt, sollte im Tierexperiment geprüft werden, ob sich hinsichtlich der Wirkungen des Coffeins auf die Funktion des Herzens und dessen energieliefernden Stoffwechsel quantitative Unterschiede zwischen verschiedenen alten Versuchstieren nachweisen lassen. Wir untersuchten daher bei 2 und 15—18 Monate alten Ratten die Wirkung von Coffein auf Herzfrequenz und Blutdruck und berechneten aus: mittlerem Blutdruck · 1/Frequenz nach Bretschneider [1] annäherungsweise die Herzarbeit. Weiterhin wurden als Parameter für den cytoplasmatischen Redoxstatus der Lactat/Pyruvat-Quotient [2] sowie als Maß für die Verfügbarkeit von chemischer Energie der ATP- und Kreatinphosphatgehalt des Herzens bestimmt.

Methodik

Unsere Untersuchungen führten wir an Ratten des Stammes Sprague Dawley durch, die bei einer Raumtemperatur von 24° C in großen Boxen mit Trinkwasser ad libitum und Standarddiät Altromin R® (Altromin GmbH, Lage Lippe) gehalten wurden. Für die Versuche wurden ebenso geschlechtsreife, 2 Monate alte, sowie 15—18 Monate alte Tiere verwendet. Die Tiere wurden mit 1,2 g/kg Urethan ip. narkotisiert, auf einen geheizten Operationstisch gebracht und nach Luftröhrenschnitt mit einer Starling'schen Beatmungspumpe (Fa. Braun, Melsungen) künstlich beatmet.

Alte und junge Versuchstiere wurden in 2 Gruppen aufgeteilt, von denen die eine jeweils 40 mg/kg Coffein iv., berechnet als Base in Form von Coffeinum Natrium benzoicum DAB. 7, die andere (Kontrollgruppe) eine gleich große Flüssigkeitsmenge 0,9% NaCl (0,1 ml pro 100 g) iv. erhielt.

Der Blutdruck wurde als Mitteldruck in der A. carotis mit einem Statham Element (Statham Miniature Blood Pressure Transducer Model SP 37) über das Druckmeßgerät Hellige Programm 19 10 min vor und bis 30 min nach der Injektion von Coffein bzw. 0,9% NaCl kontinuierlich gemessen. Gleichzeitig wurde die Herzfrequenz mit dem Eka-Puls-Gerät (Fa. Hugo Sachs Elektronik, Hugstetten) über subcutan liegende Elektroden bestimmt. Blutdruck und Herzfrequenz wurden auf dem 3-Kanal-Schreiber der Fa. Hellige Freiburg registriert.

30 min nach Verabreichung von Coffein bzw. 0,9% NaCl wurde der Thorax geöffnet und das Herz zwischen in flüssiger Luft gekühlten Aluminiumblöcken eingefroren. Nach Entfernen von anhaftendem gefrorenem Blut wurde das Gewebe mit 4 Teilen 3,5%iger gefrorener Perchlorsäure pulverisiert und anschließend das auftauende Gemisch bei 0° C abzentrifugiert. In dem aufgetauten Überstand bestimmten wir Adenosintriphosphat (ATP) und Kreatinphosphat nach

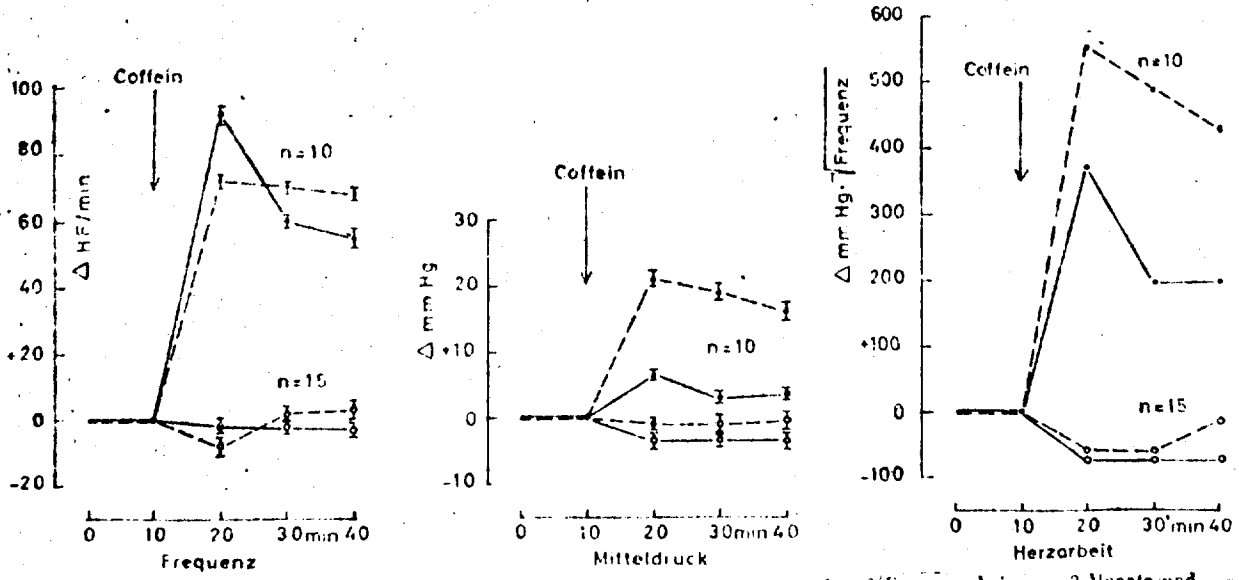

Abb. 1: Änderung von Herzfrequenz, mittlerem Blutdruck und Produkt: Mittlerer Blutdruck · 1/Frequenz bei — — 2 Monate und — — — 15—18 Monate alten Ratten nach iv. Zufuhr von —•— 40 mg/kg Coffein und —○— 0,1 ml/100 g 0,9% NaCl. Ausgangswerte: Frequenz jung: 322, alt: 345. Mitteldruck jung: 63, alt: 60 mm Hg.

Lamprecht u. Stein [6], Pyruvat*) nach Bücher (unveröffentlicht) und Lactat*) nach Scholz, Schmitz, Bücher u. Lampen [9].

Die in Abb. 1 angegebenen Werte stellen die Abweichung vom Ausgangswert nach Zufuhr von Coffein bzw. 0,9% NaCl dar. Sie sind Mittelwerte aus der jeweils angegebenen Zahl von Versuchen und deren mittlere Streuung (SEM). Die Werte der Abb. 2 und 3 sind ebenfalls Mittelwerte und deren mittlere Streuung (SEM), berechnet nach dem t-Test von Student aus der jeweils angegebenen Zahl von Einzelversuchen.

Versuchsergebnisse und Diskussion

Wie die Abb. 1 zeigt, finden sich nach iv. Zufuhr von Coffein altersabhängige Unterschiede in dessen Wirkung auf Herzfrequenz und Blutdruck. 10 min nach Verabreichung von Coffein kommt es sowohl bei den jungen als auch bei den älteren Tieren zum Anstieg der Herzfrequenz. Während die Frequenz bei den jungen Tieren bereits 20 min nach Coffeingabe wieder abnimmt bleibt sie bei älteren Tieren über die gesamte Versuchszeit von 30 min in etwa gleichem Umfange erhöht. Der Blutdruck nimmt bei den jungen Tieren nur geringfügig zu. Diese Drucksteigerung ist darüber hinaus relativ flüchtig und dauert nur 10 min. Bei den älteren Tieren ist sie dagegen wesentlich stärker ausgeprägt und auch von längerer Dauer. Berechnet man aus: Mittlerer Blutdruck · $\sqrt{\text{Frequenz}}$ annäherungsweise die Herzarbeit [1], so nimmt diese, wie die Abb. 1 zeigt, bei den älteren Tieren deutlich stärker zu als bei den jungen.

bei diesen Tieren Ausdruck einer höheren Sauerstoffausschöpfung aus dem Herzmuskel sein.

Coronardurchblutung und intrazellulärer Redoxstatus stehen derart miteinander in Beziehung, daß bei Abnahme des Sauerstoffdruckes im Gewebe, die mit einer Zunahme des Redox-Quotienten einhergeht, die Durchblutung des Herzmuskels gesteigert wird [7]. Daher dürfte der Anstieg des L/P-Quotienten im Herzen nach Verabreichung von Coffein im Zusammenhang mit einer Steigerung der Coronardurchblutung zu sehen sein. Letztere wird vermutlich bei den älteren Tieren, wegen des stärkeren Anstiegs des L/P-Quotienten mehr zunehmen als bei den jungen.

Bei beiden Gruppen kommt es jedoch, wie aus Abb. 3 hervorgeht, zu einer etwa gleich großen Abnahme von ATP und Kreatininphosphat im Herzen. Diese Befunde stehen im Einklang mit den Ergebnissen anderer Autoren nach Zufuhr von Methylxanthinen [3, 9], Isoprenalin*) [4] und Orciprenalin**) (Amon, unveröff.). Da alle genannten Stoffe neben der Abnahme von ATP und Kreatinphosphat auch zu einer Steigerung der Herztätigkeit führen, ist anzunehmen, daß die Verminderung energiereicher Phosphorsäureester nicht auf einer gestörten Energiegewinnung beruht, sondern

*) N-Isopropyl-noradrenalin sulfat.
**) 1-(3,5-Dihydroxyphenyl)-2-isopropylaminoäthanol-sulfat.

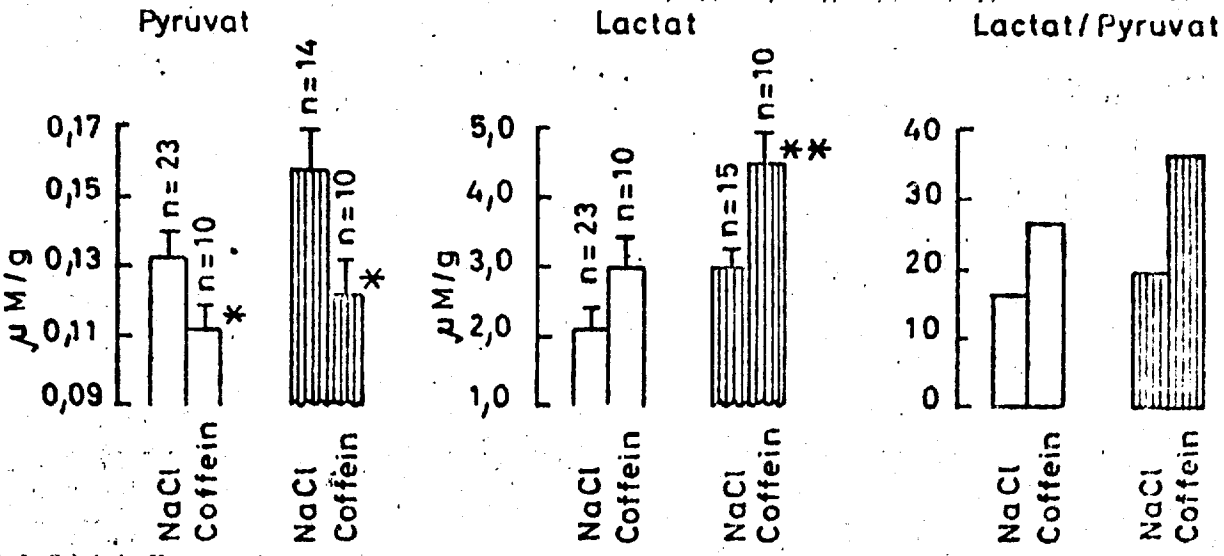

Abb. 2: Gehalt des Herzens an Pyruvat und Lactat sowie Lactat/Pyruvat-Quotient 30 min nach iv. Verabreichung von 40 mg/kg Coffein bzw. 0,1 ml/100 g 0,9% NaCl an 2 Monate und 15–18 Monate alten Ratten [ ] 2 Monate, [ ] 15–18 Monate alte Ratten. *) $P < 0,05$; **) $P < 0,01$.

Altersabhängige Unterschiede finden sich nach Zufuhr von Coffein auch im cytoplasmatischen Redoxstatus des Herzmuskels. Wie die Abb. 2 zeigt, kommt es bereits bei den jungen Versuchstieren zur Zunahme des Lactat/Pyruvat(L/P)-Quotienten um 10,8. Noch deutlicher ist der Anstieg bei den älteren mit 17,0. Die Zunahme des L/P-Quot. beruht, wie aus Abb. 2 weiter hervorgeht, sowohl auf einer Abnahme des Pyruvatgehaltes als auch auf einer Vermehrung des Lactatgehaltes im Herzen. Der bei den jungen und älteren Tieren zu beobachtende Anstieg des L/P-Quot. und damit des Red./Ox.-Quot. hat seine Ursache vermutlich darin, daß infolge größerer Herzarbeit nach Coffein die Sauerstoffausschöpfung aus dem Herzmuskelgewebe ansteigt, da Zunahme der Herzarbeit und Sauerstoffverbrauch des Herzmuskels miteinander parallel gehen [8]. Da bei den älteren Tieren die vom Herzen zu leistende Arbeit in stärkerem Umfang zunimmt als bei den jungen, dürfte der größere Anstieg des L/P-Quot.

*) Biochemica Test Combination, Boehringer, Mannheim.

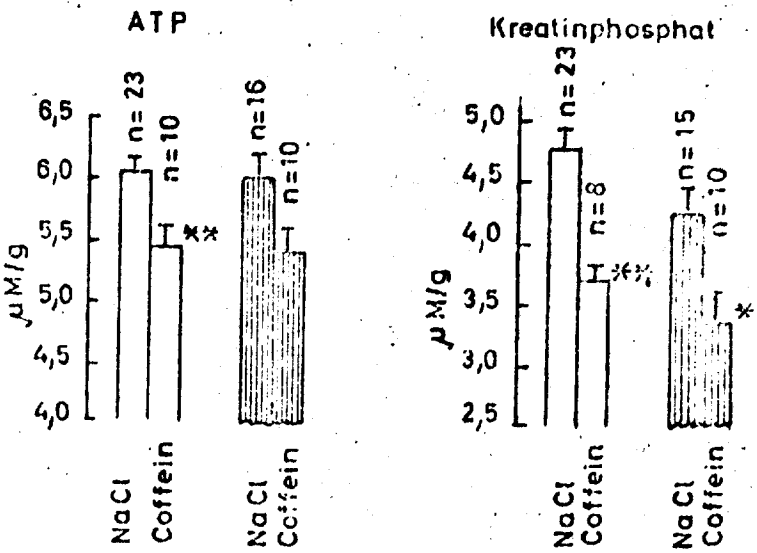

Abb. 3: Gehalt des Herzens an ATP und Kreatinphosphat 30 min nach iv. Verabreichung von 40 mg/kg Coffein bzw. 0,1 ml/100 g 0,9% NaCl an 2 Monate ([ ]) und 15–18 Monate ([ ]) alte Ratten. * $P < 0,05$; ** $P < 0,01$.

daß sich der Gehalt an energiereichen Phosphaten innerhalb eines physiologischen Bereiches auf ein niedrigeres Niveau einstellt.

Zusammenfassung

Nach iv. Verabreichung von 40 mg/kg Coffein lassen sich bei narkotisierten Ratten altersabhängige Unterschiede in dessen Wirkung auf Funktion und Stoffwechsel des Herzens nachweisen.

Herzfrequenz und vor allem Blutdruck und die aus Mitteldruck · $\sqrt{}$ Frequenz berechnete Herzarbeit nehmen bei 15–18 Monate alten Ratten stärker zu als bei 2 Monate alten.

Der Lactat/Pyruvat (L/P)-Quotient des Herzens steigt bei den älteren Tieren um 17,0, dagegen bei den jungen nur um 10,8. Der bei den älteren Tieren höhere Anstieg des L/P-Quotienten wird infolge der größeren Zunahme der Herzarbeit auf eine stärkere Sauerstoffausschöpfung aus dem Gewebe zurückgeführt.

Dagegen finden sich keine altersabhängigen Unterschiede in der Wirkung des Coffeins auf den Gehalt des Herzens an Adenosintriphosphat und Kreatinphosphat. Beide nehmen bei jungen und älteren Tieren in gleichem Umfange ab.

Frl. E. Frühauf danken wir für die gewissenhafte technische Mitarbeit.

Summary

The Role of Age in the Effects of Caffeine on Function and Metabolism of the Heart

Depending on the age caffeine (40 mg/kg i.v.) leads to quantitatively different changes of the function and the metabolism of the heart of anaesthetized rats.

Heart rate and especially blood pressure and cardiac work, which was calculated from mean blood pressure · $\sqrt{}$ heart rate increase more strongly in 15—18 months old rats than in 2 months old animals.

The lactate/pyruvate ratio of the heart is increased by 17.0 in the older animals and by 10.8 in the younger ones. The stronger increase of the lactate/pyruvate ratio in the older animals is supposed to be the consequence of a higher extraction of oxygen from the cardiac muscle because of the stronger increase of cardiac work.

On the other hand there are no age dependent differences in the action of caffeine on the ATP and creatine phosphate content in the heart. In young and older rats both are decreased to the same extent.

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PLASMA AND URINE CORTISOL BEHAVIOR AND FAT MOBILIZATION IN MAN AFTER COFFEE INGESTION

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ABSTRACT

The effect of coffee on plasma and urinary cortisol, blood glucose, plasma free fatty acid and glycerol levels was studied in 30 normal subjects. Four hours after the ingestion of coffee there was no effect on fasting blood glucose but an increase was observed in plasma free fatty acids and glycerol and in urinary cortisol excretion. The normal decrease in plasma cortisol between 8 AM and 12 noon was prevented. The effect of coffee was greater in the younger subjects (15 to 40 years) than in the older groups (41 to 70 years) irrespective of sex. On the basis of these results the evidence for the general stimulatory effect of coffee on the suprarenal gland is confirmed and extended. The associated lipid mobilization and its possible atherogenic influence are believed to be of minor importance, especially in the older age group.

Following coffee or caffein administration, an increased level of urinary and plasma catecholamines (1, 3) and an increased urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA) (4) have been observed. In man, coffee ingestion increases the urinary output of 11-hydroxycorticosteroids (11-OHCS), and in dogs an increased plasma 11-OHCS level has been demonstrated after caffein administration (5).

From these reports it is not clear whether coffee or caffein produces "stress-like" effect, or only slightly enhances the suprarenal output. Therefore, the problem was presently investigated by measuring both plasma and urinary cortisol levels, with particular attention to age and the investigated subjects.

MATERIALS AND METHODS

Thirty normal subjects, 21 males and nine females, were studied; their ages varied from 15 to 70 years. All subjects were hospitalized in our department, where full clinical and laboratory investigations did not detect any pathological process. The values of hematocrit, erythrocyte sedimentation rate, serum protein, blood urea nitrogen, creatinine clearance, blood fasting glucose, serum sodium, chloride, potassium, and protein bound iodine were all within normal range. The subjects were accustomed to drink not less than two cups of nondecaffeinated coffee daily.

The experimental procedure was begun at 8 AM and concluded at 1 PM on patients fasting for 12 hr. Blood samples were taken 1 hr before coffee or water administration (-60 min) and at 0, 15, 30, 45, 60, 120, 180 and 240 min after coffee ingestion. Blood was collected on EDTA for the determination of free fatty acids (FFA) and glyce-

TABLE 1. *Plasma FFA, glycerol, cortisol and blood glucose levels after ingestion of coffee or water*

	Time (min)							
Measured parameter	*-60*	*0*	*15*	*30*	*60*	*120*	*180*	*240*
FFA, μEq/liter								
Coffee	484 ± 106	477 ± 103	542 ± 129	613 ± 164	688 ± 170	739 ± 192	748 ± 239	697 ± 227
Water	491 ± 96	485 ± 99	487 ± 104	494 ± 114	490 ± 101	499 ± 98	503 ± 100	506 ± 101
P	> 0.05	0.05	> 0.05	0.01	0.001	0.001	0.001	0.001
Glycerol, μmoles/liter								
Coffee	740 ± 163	750 ± 163	—	—	869 ± 173	989 ± 217	1,065 ± 260	1,065 ± 260
Water	728 ± 141	728 ± 141	—	—	728 ± 152	760 ± 152	750 ± 152	761 ± 152
P	> 0.05	> 0.05	—		0.001	0.001	0.001	0.001
Cortisol, μg/100 ml								
Coffee	15.3 ± 2.1	13.5 ± 2.3	14.5 ± 2.9	16.8 ± 3.7	18.0 ± 3.8	19.4 ± 4.0	18.4 ± 3.4	17.1 ± 3.9
Water	15.5 ± 1.6	14.5 ± 1.6	14.2 ± 1.9	13.6 ± 1.8	13.1 ± 1.7	12.5 ± 1.5	11.9 ± 1.2	12.3 ± 1.2
P	> 0.05	0.05	0.05	0.01	0.001	0.001	0.001	0.001
Blood glucose, mg/100 ml								
Coffee	97 ± 11	94 ± 12	—	—	93 ± 11	94 ± 8	90 ± 11	91 ± 10
Water	95 ± 8	93 ± 10	—	—	92 ± 8	91 ± 7	90 ± 8	89 ± 8
P	> 0.05	> 0.05	—		> 0.05	> 0.05	> 0.05	> 0.05

Values are means ± SD for 30 subjects.

erol, on heparin for cortisol determination and on EDTA and potassium fluoride for glucose analysis.

Coffee or water was ingested during the time interval from 0 to +15 min. Nondecaffeinated coffee (8.5 g of instant coffee, equivalent to 250 mg of caffein) was given in a volume of 500 ml of water. The caffein content of the coffee was determined by the method of Polzella (6). As a control, 500 ml of water were given to the same subjects. Coffee and water tests were randomized and performed within not less than five days.

During the 4-hr test the patients were kept in bed; they did not drink, eat or smoke. A saline solution was infused through the indwelling needle. During the test an ECG tracing was monitored.

The patients were asked to empty their bladders at time 0; urine passed up to 240 min was then collected and its total volume and creatinine and cortisol contents (7) were determined.

In each blood sample, levels of blood glucose

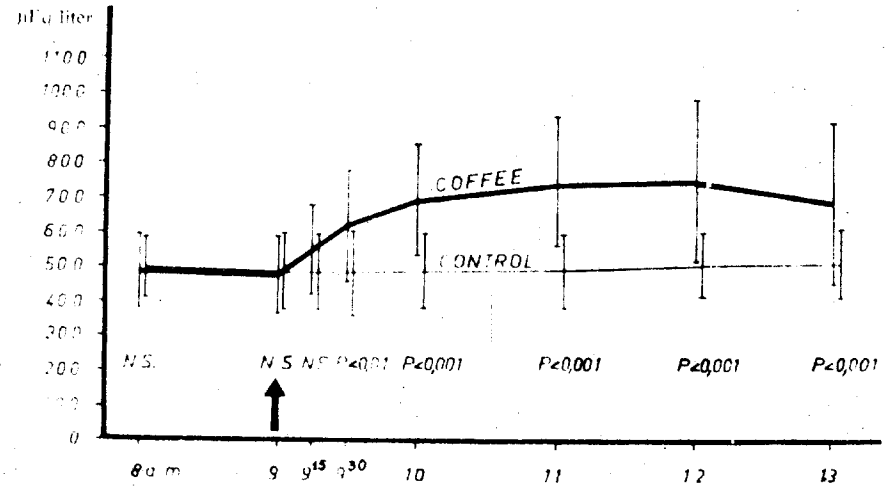

FIG. 1. Plasma FFA changes following coffee ingestion in 30 normal people.

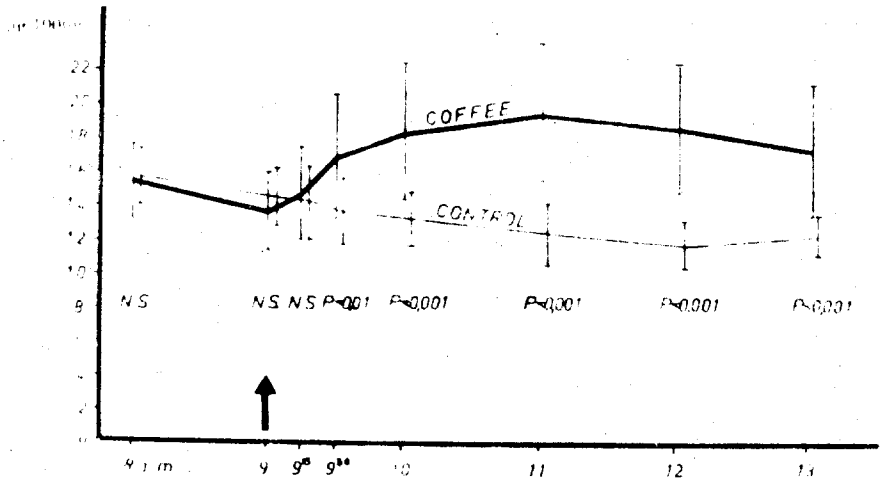

FIG. 2. Plasma cortisol changes following coffee ingestion in 30 normal people.

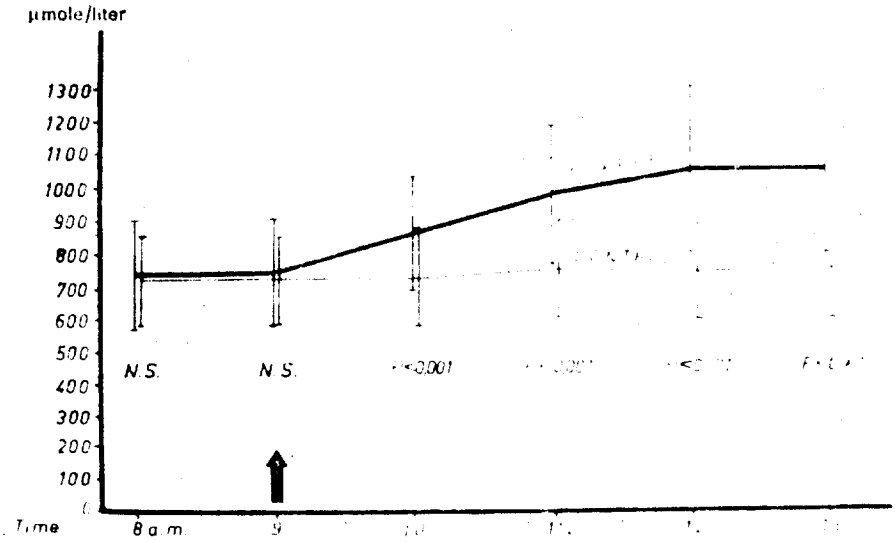

FIG. 3. Plasma glycerol changes following coffee ingestion in 50 normal people

TABLE 2. *Maximal variations in plasma FFA, and glycerol, cortisol and urinary cortisol, urinary volume and creatinine clearance after ingestion of coffee*

Measured parameter	*All subjects*	*Group I (25 to 40 years)*	*Group II (41 to 55 years)*	*Group III (56 to 70 years)*
	Subjects			
FFA, µl-eq/liter				
Coffee	+74.3 ± 22.9	+96.1 ± 21.2	+63.[illegible] ± 18.0	+63.0 ± 10.0
Water	+5.4 ± 4.0	+7.6 ± 4.3	+4.8 ± 3.9	+3.2 ± 3.7
P	0.001	0.001	0.001	0.001
Glycerol, µmoles/liter				
Coffee	+51.4 ± 12.9	+57.7 ± 16.7	+47.1 ± 12.6	+49.5 ± 9.1
Water	+4.9 ± 3.4	+4.3 ± 3.3	+5.3 ± 4.2	+5.0 ± 4.6
P	0.001	0.001	0.001	0.001
Plasma cortisol, µg/100 ml				
Coffee	+55.4 ± 20.7	+67.6 ± 23.6	+50.5 ± 21.7	+48.2 ± 19.0
Water	24.7 ± 11.2	26.2 ± 9.1	25.0 ± 7.3	22.7 ± 16.1
P	0.001	0.001	0.001	0.001
Urinary cortisol, µg[a]				
Coffee	109 ± 24	115 ± 29	100 ± 26	113 ± 13
Water	83 ± 24	79 ± 25	80 ± 29	90 ± 21
P	0.01	0.01	0.01	0.01
Urinary volume, ml[a]				
Coffee	686 ± 344	964 ± 323	512 ± 236	522 ± 301
Water	634 ± 318	833 ± 247	491 ± 173	589 ± 413
P	0.05	0.05	0.05	0.05
Creatinine clearance, ml				
Coffee	114 ± 46	127 ± 57	112 ± 49	103 ± 27
Water	108 ± 39	129 ± 83	100 ± 28	94 ± 26
P	0.05	0.05	0.05	0.05

[a] Excreted during the 240 min of the test.
The variations are expressed as percent of the basal values and given as means ± S.D. The urinary cortisol and creatinine output and urinary volume are referred to in absolute values.
A comparison between the data obtained in the three groups shows a significant difference ($P < 0.01$) for FFA, (both matching groups I and II, and I and III) and for cortisol (between I and II, and I and III). All other differences are not significant.

(8) and plasma FFA (9), glycerol (10), cortisol (7) and creatinine (11) were determined.

RESULTS

After coffee ingestion a significant increase in plasma FFA and cortisol levels was observed at 30, 60, 120, 180 and 240 min (Table 1; Fig. 1 and 2). Urinary cortisol output was also significantly increased (Table 2). Plasma glycerol rose, too, at 60 min; maximal levels were reached at 180 and 240 min (Table 1; Fig. 3). There was no appreciable change in blood glucose levels (Table 1).

The data were expressed as percentage maximal variations (Table 2). Every constituent investigated rose significantly, except for urinary volume and creatinine clearance. The increase was statistically significant in all patients considered as one group, and in patients grouped according to age irrespective of sex.

By matching the data in the patients according to age groups, a greater increase of plasma FFA and cortisol levels was shown to take place in the younger subjects in Group 1 than in those in Groups 2 and 3 (Table 2). No age-dependent differences were found with respect to other constituents.

DISCUSSION

Recent investigations have given rise to the view that coffee ingestion has a noxious effect (1-5). A relationship between coffee intake and development of ischemic heart disease has been suggested (12, 13).

On the basis of the observed increase in plasma lipid and lipoprotein levels an atherogenic effect has been attributed to coffee (14). This effect on serum lipids appears to be mediated through the caffein-induced catecholamine secretion and FFA release (1-4). Catecholamines, besides having hemodynamic effects, play a fundamental role as lipolytic hormones (15).

Until now only Bellet's group has studied the behavior of glucocorticoids after coffee (5). These authors observed an increased output of urinary 11-OCHS in man, and an increased level of plasma 11-OCHS in blood samples taken from the phrenic abdominal vein of dogs. These authors believe that coffee produces a "stress" by increasing the secretion of both cortical and medullary suprarenal hormones, with a consequent peripheral lipolytic effect which promotes the rise of plasma lipids and lipoproteins. Some authors have reported that coffee has a diabetogenic effect, which causes an increased blood glucose level, gives a worse "K" value of glucose removal after an i.v. glucose tolerance test and retards insulin secretion (16-20). Only Bellet and co-workers (21) have observed a reduction in blood glucose levels after an oral glucose load following oral or i.m. caffein administration (21).

Our data confirm those of Bellet et al. (5) indicating an increased 11-OCHS urinary output after coffee ingestion. In addition, we have demonstrated that, in man, coffee increases plasma cortisol levels. Our results may be interpreted to mean that coffee ingestion actually prevents the decrease in plasma cortisol level that normally occurs during the morning hours after 8 AM (22) rather than that it causes a sharp increase in cortisol output. The observed behavior of plasma cortisol differs considerably from the sharp increase which occurs during the stress (23).

The fact that in our experiments fasting blood glucose levels did not change significantly does not necessarily contradict previous results (16-20), in which tolerance to glucose after coffee was studied after a glucose load.

Our data point to a strong lipolytic effect of coffee ingestion, as evidenced not only by the rise in plasma FFA levels previously reported by Bellet et al. (24), but also by the increase in plasma glycerol levels. Glycerol appears to be a more direct index of lipolysis than plasma FFA (25). The increase in lipoly-

sis may be linked to the increased catecholamine secretion (1-3) as well as to the retarded insulin secretion (20).

The possibility that high FFA levels after coffee may be atherogenic, on account of ensuing hyperlipoproteinemia, was postulated by Bellet et al. Persistent hyperlipoproteinemia after coffee may however apply only to a class of particularly susceptible people, characterized by an increased synthesis or a deficient removal of plasma lipids. In normal people, coffee seems to represent a transient supply of additional substrate especially when carbohydrates are not available.

Analysis according to age showed that the responses of plasma FFA and glycerol and of plasma and urinary cortisol were significantly greater in the younger than in the older subjects. This suggests that the responses to coffee ingestion are related to the sensitivity of the suprarenal gland. Thus, in older people the lipid-mobilizing effect of coffee and its possible consequences seem to be of lesser importance.

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THE FATE OF CAFFEINE IN MAN AND A METHOD FOR ITS ESTIMATION IN BIOLOGICAL MATERIAL

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Caffeine is extensively used as a therapeutic agent and is widely ingested in the form of caffeine containing beverages, yet little is known concerning its fate in the body. The development of a simple and sensitive method for the estimation of caffeine in biological materials has permitted studies of its absorption, excretion, distribution and rate of biotransformation in man.

CHEMICAL METHOD. *The estimation of caffeine.* Older methods for the determination of caffeine in biological material involve extraction into chloroform, followed by assay of the drug by its nitrogen content (Kunz, 1935) or by a color-producing murexide reaction (Tanaka and Ohkubo, 1937). These methods are insensitive and inaccurate. Recently, Ishler *et al.* (1948) described a sensitive method, using ultraviolet spectrophotometry, for the estimation of caffeine in coffee preparations. This method was applied by Fisher *et al.* (1949) to the estimation of caffeine in urine. In this method caffeine is extracted from the biological material with chloroform, which also extracts other methyl xanthines and interfering normal constituents of biological materials.

In the following procedure, caffeine is isolated from biological material at pH 7-8 by extraction into benzene. The compound is then returned to an aqueous phase by shaking with 5 *N* HCl and its absorption in the ultraviolet is measured at 273 mμ. The relatively nonpolar solvent benzene was chosen as the solvent least likely to extract metabolic products of caffeine and normal constituents of urine according to principles previous described (Brodie *et al.*, 1947). The extraction is augmented by saturating the aqueous phase with sodium chloride. A small amount of interfering blank material in urine and tissues which is also extracted into benzene is almost entirely removed by shaking the solvent extract with dilute NaOH. Since caffeine is basic it can be extracted from the benzene phase into 5 *N* HCl. This effectively separates it from methylated xanthines such as theobromine and theophylline.

Procedure for plasma. Pipet 1 to 5 ml. of plasma into a 60 ml. glass-stoppered bottle containing 40 ml. of benzene[2] and about 3 gm. of sodium chloride. Shake for 20 minutes. Centrifuge the bottle and pipet 30 ml. of the benzene phase into a 60 ml. glass-stoppered bottle[3] containing 4 ml. of 5 *N* HCl. Shake for 5 minutes, transfer 3 ml. of the acid phase to a quartz cuvet. Determine the optical density at 273 mμ in a Beckman DU spectrophotometer.[4]

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[1] Part of the material in this paper was taken from a thesis submitted to the Chemistry Department of the Graduate School of Georgetown University, in partial fulfillment of the requirements for the degree of Master of Science.

[2] Benzene, reagent grade, is purified by successive washings with ⅕ volume of 1 *N* NaOH, 1 *N* HCl, and twice with water.

[3] Glassware used in this and subsequent steps in the chemical procedure is immersed in 5 *N* HCl for 2 hours and thoroughly rinsed with distilled water. The quartz cuvets are similarly treated.

[4] The optical density reading of 5 *N* HCl in a quartz cuvet slowly increases with time. To minimize this potential error, the optical density readings should be measured within a few minutes after the extraction of the caffeine into the acid phase.

The instrument is set at 100 per cent transmission with distilled water. An optical density of about 0.200 is obtained when 25 microgm. of caffeine are assayed by this procedure. Standards are prepared by dilution of a stock caffeine solution with 5 *N* HCl.

Urine and tissues. Pipet 3 ml. of urine or tissue homogenate,[5] adjusted to pH 7-8, into a 60 ml. glass-stoppered bottle containing 40 ml. of benzene and about 3 gm. of sodium chloride. Shake for 20 minutes. Centrifuge the bottle and transfer as much of the benzene phase as possible to a 60 ml. glass-stoppered bottle containing 3 ml. of 0.1 *N* NaOH saturated with sodium chloride. Shake for 5 minutes and centrifuge. Pipet 30 ml. of the benzene phase into a 60 ml. glass-stoppered bottle containing 4 ml. of 5 *N* HCl and continue as described for the estimation of caffeine in plasma.

Caffeine added to biological material in amounts from 5 to 50 microgm. were recovered with adequate precision (98 ± 3 per cent).

Appraisal of specificity. There is a small blank equivalent to 0.1-0.5 microgm. of caffeine per ml. of normal plasma and 0.5 to 2.0 microgm. per ml. of urine or per gram of organ tissue. The possible interference of metabolic products of the drug in the plasma procedure was examined by the technique of comparative distribution ratios (Brodie *et al.*, 1947). Plasma was obtained two hours after 7 mgm. per kgm. of caffeine was administered intravenously to two human subjects. The apparent caffeine was extracted from the pooled plasma in the same manner as described in the procedure. The distribution ratios of the apparent caffeine were compared with those of authentic caffeine in a series of two phase systems consisting of water at pH 7 and benzene and petroleum ether in various proportions. The results indicate that the two substances possess the same solubility characteristics and were presumably the same compound (table 1).

TABLE 1

Distribution of caffeine and apparent caffeine between water and various benzene-petroleum ether mixtures

The apparent caffeine from the pooled plasma of two subjects receiving caffeine was extracted into benzene as described under the chemical method. The drug was returned to 5 *N* HCl. The acid extract was adjusted with NaOH to pH 7. Aliquots of this solution and of an authentic solution of caffeine similarly adjusted to pH 7 were shaken with 4 volumes of the various benzene petroleum ether mixtures. The distribution is expressed as the ratio of the amount of compound in the organic phase to total compound.

PER CENT PETROLEUM ETHER IN BENZENE	APPARENT CAFFEINE FROM PLASMA	AUTHENTIC CAFFEINE
0	0.89	0.92
25	0.81	0.84
50	0.60	0.62
63	0.45	0.46
75	0.27	0.27

EXPERIMENTAL. *Urinary excretion of caffeine.* Three male subjects each received 500 mgm. of caffeine[6] intravenously and urine was collected for the subsequent 24 hours. Only 0.5 to 1.5 per cent of the administered caffeine was excreted unchanged, indicating that the drug had undergone almost complete transformation in the body.

[5] Organ tissues are prepared for analysis by emulsification in water as described in a previous paper (Brodie *et al.*, 1947).

[6] The caffeine was administered in this and in subsequent experiments as caffeine-sodium benzoate.

Absorption of caffeine. Information was obtained concerning the part played by absorption from the gastrointestinal tract in the physiological disposition of caffeine. Plasma levels of the drug were compared after oral and intravenous administration of 7 mgm. per kgm. of caffeine in three male subjects. After oral administration, peak plasma levels of about 10 mgm. per liter occurred in about one hour after which the levels closely approximated those following the intravenous administration (fig. 1). Absorption from the gastrointestinal tract is thus rapid and essentially complete.

Distribution of caffeine in tissues. The extent to which caffeine in plasma is bound to proteins was determined by dialysis at 37°C. for 18 hours through

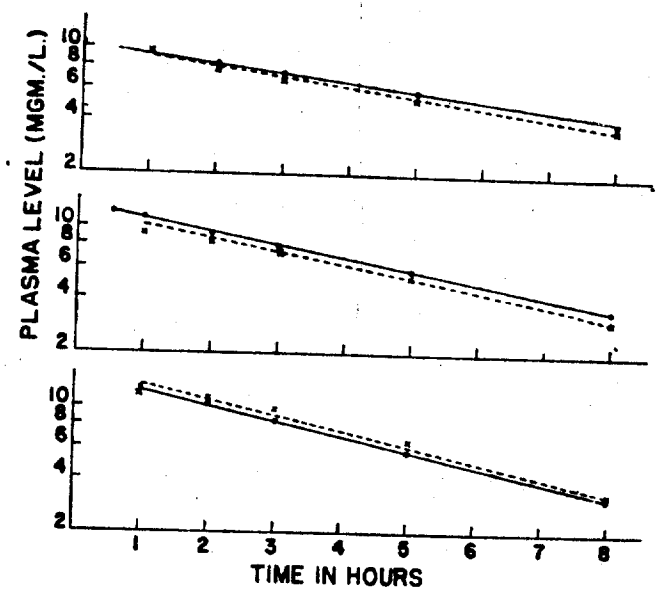

FIG. 1. Plasma levels of caffeine after the oral (solid line) and intravenous (dashed line) administration of 7 mgm. per kgm. of caffeine to three subjects.

Visking membranes against isotonic phosphate buffer at pH 7.4. In these experiments the drug was added directly to plasma. At plasma levels ranging from 9 to 45 mgm. per liter, about 15 per cent of the caffeine was found to be bound to the non-diffusible constituents of the plasma.

The distribution of caffeine was examined in representative tissues of a dog 3 hours after the intraperitoneal injection of 84 mgm. per kgm. of the drug. The animal was sacrificed by an intravenous injection of air and the tissues were sampled immediately afterwards. Tissue water was determined by drying to constant weight at 95–100°C. The tissue concentration of caffeine was measured and calculated in terms of tissue water. The caffeine concentration in the water of various tissues was found to be nearly the same as that in plasma water (table 2), indicating that the drug is distributed in tissues in approximate proportion to their water content. The concentration of caffeine in cerebrospinal fluid was

found to be almost the same as that in plasma water, suggesting that there is little hindrance to the passage of the drug across the blood-brain barrier.

The rate of disappearance of intravenously administered caffeine from plasma. Twelve male subjects were given 7 mgm. per kgm. of caffeine, administered intravenously, and the drug plasma levels were measured at various time intervals. In all cases the logarithm of the plasma concentration when plotted against

TABLE 2

The distribution of caffeine in dog

The dog received 84 mgm. per kgm. of caffeine intravenously. The tissues were examined 3 hours after the administration of the drug.

TISSUE	CAFFEINE IN TISSUE WATER *mgm./kgm.*	RATIO: $\frac{\text{Tissue water caffeine}}{\text{Plasma water caffeine}}$
Plasma	82.7	1.0
Red blood cells	83.0	1.0
Cerebrospinal fluid	78.2	0.95
Liver	91.7	1.11
Lung	71.5	0.87
Heart	77.0	0.93
Muscle	71.0	0.86
Kidney	82.1	0.99
Spleen	73.2	0.89
Brain	77.2	0.93

TABLE 3

Plasma levels of caffeine after coffee drinking

Two cups of coffee taken at 8:00 a.m., 12 noon, 3:00 p.m., and 6:00 p.m. Blood samples were drawn at 8:00 a.m. (B_0), 9:00 a.m. (B_1), 1:00 p.m. (B_2), 7:00 p.m. (B_3), and the following morning at 8:00 a.m. (B_4). No caffeine containing beverages were taken 24 hours prior to the experiment or during the interval between B_2 and B_4.

SUBJECT	PLASMA CAFFEINE CONCENTRATION MGM./L.				
	B_0	B_1	B_2	B_3	B_4
1	0.4	2.7	3.5	4.8	0.3
2	0.9	1.5	1.9	4.0	0.2
3	0.5	0.7	1.3	4.2	0.6
4	0.4	1.9	2.8	3.9	0.7
5	0.03	1.6	2.1	3.6	0.5

time yielded a straight line indicating that caffeine disappears at a rate proportional to its plasma concentration (fig. 1, typical examples). Since negligible amounts of caffeine are excreted in urine and the drug is not localized in tissues, this decline reflects its rate of biotransformation. The biologic half-life, that is the time required for the plasma level to fall to half its value, was found to be fairly uniform from subject to subject, averaging 3.5 hours and ranging from 2.5 to 4.5 hours (12 to 22 per cent disappearance per hour).

Biotransformation of caffeine in dogs. Plasma levels and urinary excretion of caffeine were studied in dogs following the intravenous administration of 4

mgm. per kgm. of the drug. In this species also the drug was almost entirely metabolized, little or none being excreted in the urine. The biologic half-life in the dog averaged 5 hours, a value about the same as that found for man.

Accumulation of caffeine after repeated coffee drinking. In view of the widespread consumption of coffee it was of interest to determine whether caffeine accumulates in the body after the repeated drinking of coffee. Plasma levels of caffeine were measured in five human subjects who has consumed eight cups of coffee[7] over a period of seven hours. It is apparent that after the repeated intake of coffee considerable amounts of caffeine accumulate in the body (table 3). The total amount of caffeine in the body may be calculated by multiplying the total body water by the concentration of drug in plasma water, since it has been shown (*vide supra*) that caffeine is approximately evenly distributed in body water. Assuming a total body water content of 55 per cent (Soberman *et al.*, 1950) it can be estimated that about 180 mgm. of the drug were present in the body one hour after the last cup of coffee. This amount of caffeine is approximately equivalent to a therapeutic dose of the drug. There was no day to day accumulation of caffeine, as shown by the virtual disappearance of the drug by the following morning.

SUMMARY

A sensitive method for the estimation of caffeine in biological material is described. The method is specific in that it does not include transformation products of caffeine in the measurement.

Caffeine is rapidly and essentially completely absorbed from the gastrointestinal tract of man.

Caffeine is almost entirely transformed in man, only about 1 per cent being excreted in the urine. The rate of biotransformation is fairly uniform from subject to subject, the average half-life being 3.5 hours (15 per cent metabolized per hour). The metabolic rate of transformation in dogs is about the same as in man.

Caffeine is distributed in various tissues in approximate proportion to their water content. The drug passes rapidly into the central nervous system.

Although a considerable amount of caffeine accumulates in the body of moderately heavy coffee drinkers during the day, there is no day to day accumulation of the drug.

Acknowledgment. The authors wish to thank Dr. Victor McKusick for the administration of caffeine to the human subjects.

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[7] Soluble coffee was used in these experiments. Each cup of coffee contained about 80 mgm. of caffeine.

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CREATINURIA IN MAN FOLLOWING THE ORAL ADMINISTRATION OF CAFFEINE[1]

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An increase in the excretion of creatine has been observed in the urine of rats (1, 2) and of rabbits (3) following the parenteral injection of caffeine. In a study of the action of this drug on the metabolic processes in man we have frequently found creatine in the urine after oral administration. These observations were but one phase of a more comprehensive study of the effect of caffeine on metabolism as shown by the respiratory exchange and the urinary constituents. Data on the respiratory exchange have been reported elsewhere (4). The purpose of the present study is to show the amount of creatine and the frequency of its appearance in the urine with different doses of caffeine and to determine whether there was any relationship between creatinuria and the respiratory exchange, carbohydrate oxidation and protein metabolism.

METHOD. Three male adults served as subjects. Each customarily used coffee in moderation but abstained from all caffeine-containing beverages for twenty-four hours before the experiment. On the morning of the experiment the subject came to the laboratory in the fasting state and reclined for 30 to 45 minutes to recover from the small amount of exercise he had taken. The bladder was then emptied and the urine discarded. The subject then reclined for a basal period of 45 minutes, at the conclusion of which the urine was collected for analysis. This collection will hereafter be designated as the "post-absorptive" urine. Caffeine alkaloid dissolved in 200 cc. water at body temperature was then taken and, in the control experiments, an equal volume of water. The amount of caffeine ingested ranged from 0.6 to 6.0 mgm. per kilo body weight. The recumbent position was resumed for one hour and forty-five minutes and at the end of this time the urine was collected for analysis. The gaseous exchange was measured throughout the post-absorptive and post-ingestion periods in the manner described previously (5).

Creatine was determined by Benedict's method (6), the picric acid used in the analysis having been purified according to his directions (7). The data obtained are taken to be true values of creatine since Beard (1) using the enzyme of Miller and Dubos has shown that the chromogenic reaction in urine is specific for creatinine.

RESULTS. The hourly urinary excretion of creatine for a period of one and three-quarter hours in all the experiments is shown in table 1. When the amount of caffeine ingested was 1.8 mgm. or more per kilo body weight, creatine appeared in the urine with but few exceptions. One milligram per kilo, however,

[1] Preliminary report: Federation Proceedings, March 16, 1942, p. 4.

induced creatinuria in only 3 out of 14 experiments. In two other experiments creatine was excreted in the post-ingestion period but these are not included in the table since it was present also in the post-absorptive urine. In none of the 8 experiments with 0.6 mgm. caffeine per kilo was there any creatine in the urine. Approximately one milligram caffeine per kilo therefore appears to be the minimal amount capable of inducing creatinuria in our subjects.

Inspection of the data in table 1 shows that there was no direct relationship between the amount of creatine excreted and the amount of caffeine ingested. The average hourly excretion was slightly less with 1.8 than with 1.0 mgm. caffeine per kilo and also less with 6.0 than with 3.0 or 3.6 mgm. The maximum excretion was approximately the same with 1.0 as with 1.8 mgm. caffeine and less with 6.0 mgm. than with 3.0 and 3.6 mgm. per kilo.

Creatine excretion and the respiratory exchange. Coefficients of correlation were calculated for the hourly excretion of creatine and 1, the increase in oxygen consumption above the basal level; 2, the increase in carbon dioxide elimination; 3, the total oxygen consumption; and 4, the total carbon dioxide elimination during one and three-quarter hours after the ingestion of caffeine. There was no significant correlation between the amount of creatine excreted and the respiratory exchange in terms of either the increase above the basal or of absolute amounts of oxygen consumed or carbon dioxide eliminated. The coefficients of correlation ranged from 0 to 0.10. Furthermore, there was no relationship between the occurrence of creatinuria and the height of metabolism. Since there was an increase in oxygen consumption and carbon dioxide elimination with the larger doses of caffeine, it is obviously necessary to limit comparisons of the gaseous exchange to those experiments in which the same dosage of caffeine was used. Such comparisons showed that in the different groups of experiments with various doses of caffeine, the range of increase in oxygen consumption and carbon dioxide elimination either as absolute values or as percentages above the basal was the same whether or not creatinuria occurred.

TABLE 1

Urinary excretion of creatine after ingestion of various amounts of caffeine

CAFFEINE INGESTED PER KILO BODY WEIGHT	NUMBER OF EXPERIMENTS	NUMBER OF EXPERIMENTS WITH CREATINE IN THE URINE IN P-E PERIOD	RANGE	HOURLY EXCRETION OF CREATINE (AVG. OF ALL EXPTS.)	HOURLY EXCRETION OF CREATINE (AVG. OF EXPTS. IN WHICH CREATINE WAS EXCRETED)
mgm.			*mgm.*	*mgm.*	*mgm.*
None*	10	0		0	0
0.6	8	0		0	0
1.0	14	3	0.9–1.4	0.4	1.2
1.8	6	4	0.6–1.5	0.7	1.0
3.0	12	11	0.8–4.7	1.6	1.7
3.6	7	7	1.5–4.2	2.2	2.2
6.0	10	8	0.7–2.2	1.0	1.4

* Control experiments.

As shown elsewhere (4) a rise in the respiratory quotient occurred after the ingestion of caffeine and reached the highest levels with the largest dosage. A critical study of the respiratory quotients of the individual experiments revealed, however, that there was no relationship between the presence of creatine in the urine and the height of the respiratory quotient. As in the case of the respiratory exchange it was necessary to limit comparisons to experiments with the same dosage of caffeine. A study of the individual non-protein respiratory quotients showed that there was no uniform or significant differences between the quotients in the experiments with and without creatinuria.

Carbohydrate metabolism. The presence of creatine in the urine was not related to the amount of carbohydrate oxidized. In those experiments in which creatine appeared in the urine after the ingestion of caffeine the total carbohydrate oxidation was not appreciably different than in those in which creatinuria did not occur, or in the control experiments in which water alone was taken. After the ingestion of 3 mgm. and 6 mgm. caffeine per kilo there was a marked rise in the respiratory quotient during the first and second periods after ingestion, but this rise was due to hyperventilation and not to an increase in carbohydrate metabolism (4). It was later followed by a fall of the quotient below the base line because of a compensatory retention of carbon dioxide. Notwithstanding the hyperventilation, calculations of the amount of carbohydrate oxidized on the basis of the respiratory exchange for the entire post-ingestion period of 1¾ hours were doubtless correct inasmuch as the compensation by retention of carbon dioxide was completed at the end of this period.

Creatinuria and nitrogen excretion. The procedure followed in these experiments was the same as in numerous experiments on metabolism conducted in our laboratories. Under these conditions we have found that the nitrogen excretion is almost invariably less in the post-experimental than in the post-absorptive period. Inspection of the average excretion in the different groups of the caffeine experiments indicated a trend toward smaller differences between the post-absorptive and post-experimental excretion of nitrogen as the dosage of caffeine was increased. A statistical analysis of the data showed that in those experiments in which creatine was found in the urine the decline in nitrogen excretion during the post-experimental period was significantly less than when there was no creatine present. In the experiments with no creatine in the urine, the average hourly nitrogen excretion was 108 mgm. less in the post-experimental than in the post-absorptive period whereas in the experiments with creatinuria it was 15 mgm. less. The difference between these two values (93 mgm.) was statistically significant as it was four times the sigma of the difference.

Discussion. Since there was no relationship in these experiments between the presence or the amount of creatine in the urine and the oxygen utilization or carbon dioxide elimination, it appears that the underlying mechanism responsible for creatine excretion following the ingestion of caffeine is not related to an increase in total metabolism. In view of these findings, may not the creatinuria observed in hyperthyroidism (cf. 8, p. 217) be merely incidental and not due to the heightened metabolism?

Brentano (3) found that the injection of 2 mgm. caffeine per kilo body weight

into rabbits was followed by creatinuria which paralleled a diminution in the glycogen content of the skeletal muscles. From these and other experiments he concluded that those conditions which lead to a reduction in muscle glycogen result in the excretion of creatine. Our experiments offer no direct evidence with regard to the glycogen content of the muscle before and after the administration of caffeine, but if a diminution in muscle glycogen did occur, it was not reflected either in a rise in blood lactic acid (5) or in an increased oxidation of carbohydrate.

Creatinuria induced by the ingestion of caffeine could conceivably result from an increase in the rate of blood flow with the consequent washing out of creatine from the tissues. Although the rate of blood flow in these experiments is not known, in view of the results obtained by Beard and his associates on rats (1, 9) it may be assumed that this factor was not responsible for the creatinuria. These observers found that after parenteral injection of caffeine the increase in the amount of creatine in the urine was accompanied by an increase and not by a decrease in the creatine content of the muscle. Creatinuria could perhaps be the consequence of diuresis but this did not occur in our experiments. In all the experiments the hourly excretion of urine was less in the post-experimental than in the post-absorptive period.

The larger amount of nitrogen in the post-experimental relative to the post-absorptive urine that was found when creatinuria occurred, could have resulted from an increase in the total volume of urine excreted or from stimulation of protein metabolism. Analysis of the data showed that there was no significant difference in the total volume of urine in the experiments with and without creatinuria. In the former the average hourly excretion was 14 cc. less in the post-experimental than in the post-absorptive period, while in the latter it was 56 cc. less. The difference between these two values was only 1.8 times the sigma of the difference. The difference between the nitrogen excretion in the experiments with and without creatinuria was statistically significant. Likewise, the difference between the excretion in the control experiments with water and with caffeine when the dosage exceeded 1 mgm. per kilo was significant. It appears therefore that the smaller difference between the post-absorptive and post-experimental excretion of nitrogen associated with creatinuria must be attributed to a stimulation of protein metabolism by caffeine.

The increase in protein metabolism may have been merely coincidental with the creatinuria or may have been a causative factor. While no definite conclusion can be drawn from the data at hand, it appears probable that the effects produced by caffeine on protein metabolism and creatine formation (or elimination) may represent two unrelated actions of the drug. This conclusion is suggested by a comparison of the results of these experiments with those obtained after the ingestion of 50 grams glucose or the same amount of fructose (10, 11). Creatinuria was induced almost invariably by the ingestion of fructose but rarely by the same amount of glucose. There was, however, no increase in protein metabolism (nitrogen excretion) in those experiments in which there was creatinuria, as compared with those in which no creatine was excreted.

As stated previously, there was no correlation in these experiments with

caffeine between the amount or presence of creatine in the urine and the total amount of carbon dioxide eliminated. However, after administration of the larger doses of caffeine which usually produced creatinuria, there was a blowing off of preformed carbon dioxide which was compensated for by retention before the conclusion of the experiment (4). In the experiments with fructose there was likewise a loss of non-metabolic carbon dioxide as a result of the formation of lactic acid (5). In both instances it may be reasonably assumed that there was a temporary alkalosis of the tissues. This common factor in the experiments with caffeine and with fructose would suggest that alkalosis may operate as a fundamental mechanism in the formation or liberation of creatine. It must be admitted, however, that different mechanisms might account for creatine formation under various experimental and pathological conditions.

CONCLUSIONS

The administration of sufficiently large doses of caffeine was followed by the excretion of creatine in the urine.

The minimal dose of the drug that induced creatinuria was 1 mgm. per kilo, but with this amount creatine was excreted in only 3 out of 14 experiments. With doses of 1.8 to 6.0 mgm. caffeine per kilo, creatine was found in the urine in 30 out of 35 experiments.

The amount of creatine excreted showed no relationship to the amount of caffeine ingested.

There was no correlation between the amount of creatine in the urine and total oxygen consumption and carbon dioxide elimination; nor was there any relationship between the occurrence of creatinuria and the gaseous exchange.

The presence of creatine in the urine was not related to an increase in carbohydrate oxidation.

An increase in protein metabolism was associated with creatinuria but it appears that it was not a causative factor.

While it is recognized that different mechanisms might account for creatine formation under various experimental and pathological conditions, these experiments in conjunction with others on fructose suggest that alkalosis might play a significant rôle in some instance.

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REPRODUCTIVITY AND GROWTH OF ALBINO RATS ON A PROLONGED DAILY INTAKE OF CAFFEINE

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ONE FIGURE

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Studies made by different investigators on the effect of caffeine on reproductivity and growth have led to divergent conclusions. Stieve ('30, '31) described degenerative changes in the testicles of rabbits that had been given caffeine in a coffee brew by stomach tube for 3 to 4 weeks. These changes he believes accounted for the smaller number of offspring delivered by the females in his test group on coffee as compared with the controls given an equal amount of Kaffee Hag containing only a minute amount of caffeine. Vacca ('26) had previously reported a deleterious action of caffeine on the dog's testicle. Eichler and Mügge ('32), however, found that daily subcutaneous injections of caffeine did not result in a diminution of the reproductive capacity of albino rats over four generations. Their observations did not extend over the entire reproductive period of any one of the four generations. Hammill and Miller ('35) reported that litters of their test animals given caffeine containing beverages were of normal size. At variance with these results are the preliminary observations of Cheney ('44) indicating a reduced fecundity in rat colony studies extending over a period of 5 years.

As regards growth, some investigators (Hammill and Miller, '35; Smith and Hambourger, '36) report that prolonged

administration of caffeine retards it. Others (Stieve, '30; Eichler and Mügge, '32) found that the drug had no effect. In the experiments of Scott and Chen ('44) there was no depression in growth when the food mixture given the animals contained caffeine in a concentration as high as 0.1%.

The indecisiveness of these studies suggested the advisibility of the present investigation. Because of the extensive use of the caffeine-containing beverages, coffee and tea, the problem assumes practical interest from the nutritional point of view.

PROCEDURE

Twenty-five groups of albino rats were set apart at weaning for the experiment. Each group consisted of two males and two females from the same litter. One pair (male and female) served as test animals, the other pair as controls. All the animals were fed the same ration of commercial dog food [1] supplemented with green food and cod liver oil and were allowed to eat ad libitum. The drinking fluid to which there was free access at all times was tap water for the controls and a sweetened beverage containing caffeine for the test animals. This beverage constituted the sole fluid intake from the time of weaning to the conclusion of the experiment. It was chosen in preference to a solution of caffeine in water in order to insure as large an intake of caffeine as possible. Preliminary experimentation had revealed that the animals would drink a much larger quantity of this beverage than a solution containing the same amount of caffeine in plain water. The fluid was placed in an inverted graduated test tube provided with a drinking tip to which the animal had ready access. Various tests and observations had shown that there was no loss of fluid when the apparatus was left undisturbed and an extremely small loss when the animal was drinking. This was estimated to be considerably less than 5% of the contents of the container. The fluid intake of the adult control animals was approximately 30 ml per day

[1] Purina Dog Chow.

and that of the test animals 60 ml. This volume of the caffeine solution contained 12 mg caffeine which gave an average daily intake of approximately 40–50 mg per kilo body weight.

When the animals were 100 days old they were mated, a control male with a control female and a test male with a test female. Each pair was kept in a separate cage throughout the experiment. Records were kept on the date of birth of each litter, the number of offspring and the number of young that survived to weaning at the age of 21 days. The experiment was terminated for the individual pairs of animals when the females failed to become pregnant within 3 months after the birth of the last litter. The animals were then sacrificed and the testicles of the males removed and prepared for histological examination. The experiment was carried on continuously over 3½ years.

RESULTS

Reproductivity

The experimental data presented in table 1 show that there was considerable variation in the number of litters and number of offspring in each litter among both test and control animals. The averages were slightly higher in the test than in the control animals but the differences were not statistically significant. The critical ratio was only 1.3 times the difference.

The control animals gave birth to 105 litters containing a total of 618 offspring, or an average of 5.9 per litter; the test females delivered 135 litters containing 714 offspring, or an average of 5.3 per litter. Two of the control females produced no offspring whereas all of the females of the test group had at least one litter. There were four females in the control group and one in the test group that failed to raise a single animal to the weaning stage. 52.2% of the total number of offspring of the test animals and 53.2% of the controls survived to weaning. The average length of time between mating and delivery of the first litter was 31 days for the controls and 29 days for the test animals.

TABLE 1

Reproductivity of albino rats on a caffeine-containing beverage as their sole fluid intake, as compared with their litter mate controls on water.

	TESTS			CONTROLS		
Group number	Number of litters	Number of offspring	Number weaned	Number of litters	Number of offspring	Number weaned
1	3	29	13	1	5	0
2	5	16	0	6	40	32
3	11	71	47	6	34	22
4	4	32	20	14	74	33
5	4	28	9	1	3	0
6	4	14	1	0	0	0
7	9	32	7	6	39	18
8	11	45	33	6	45	39
9	7	50	19	3	17	8
10	9	58	37	13	76	61
11	1	8	4	2	10	6
12	4	27	22	2	8	3
13	4	23	16	3	24	9
14	1	5	5	7	44	3
15	10	42	27	4	18	16
16	4	18	4	2	9	0
17	7	32	12	6	34	24
18	3	13	9	1	8	0
19	1	8	5	2	19	5
20	6	27	17	3	18	11
21	9	30	11	3	19	5
22	5	27	10	1	9	2
23	7	52	27	7	29	12
24	3	15	10	0	0	0
25	3	12	8	6	36	20
Total	135	714	373	105	618	329
Average per group	5.4	29	15	4.2	25	13

Testicular histology

A histological examination [2] was made on the testicles of twenty-three of the test males and fifteen controls. The find-

[2] All histological studies were done by Dr. Walter Sheldon, pathologist at Grady Hospital, Atlanta, Ga.

ings on 57% of the tests and 53% of the controls were essentially negative. In the remainder there occurred certain histological changes which, however, were of the same type in both the tests and controls. None of the lesions resembled those described by Stieve in rabbit's testicles. In some of the testicles small patchy areas were observed in which the seminiferous tubules had become atrophic. These tubules were lined by a thin, irregular layer of atrophic epithelium suggesting fatty degeneration. There was no trace of spermatogenesis left. In these areas there was some increase in the interstitial cells but no appreciable thickening of the tubular basement membrane. The remaining portions of the testes were normal with normal spermatogenesis. In other testicles there was a slight general reduction in spermatogenesis but no areas of atrophy. Several showed extensive areas of tubular atrophy and fibrosis with reduced spermatogenesis in the remaining tubules. These changes may be accounted for by spontaneous physiological involution.

No relationship could be detected between the histological changes in the testicles and the number of offspring. For example, in animal no. 17 of the test group, the testicles showed fairly extensive lesions and in animal no. 20 the lesions were very slight. However, the former sired seven litters containing a total of thirty-two young, while the latter sired only six litters containing twenty-seven young. In both these instances the number of offspring was larger than in many cases in which no histological changes were seen in the testicles.

Growth

Weekly weighings were made on ten test and ten control males for 26 weeks and on an equal number of females for 11 weeks. The weighings were discontinued on the females at this time because they were then mated and became pregnant shortly thereafter. From the weight curves presented in figure 1 it is apparent that the daily ingestion of caffeine had no retarding effect on growth.

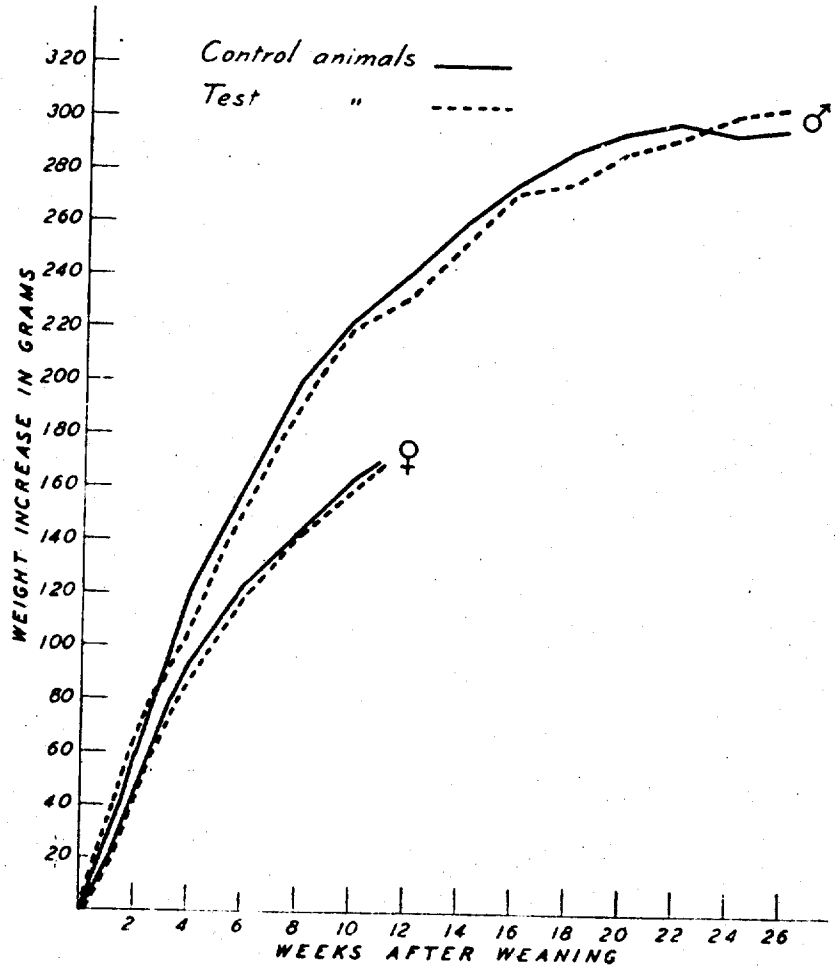

Fig. 1 Growth curves of albino rats on a caffeine containing beverage as their sole fluid intake, as compared with their litter mate controls on water.

DISCUSSION

In the experiments of Eichler and Mügge ('32) it was found that the prolonged administration of caffeine to albino rats produced no diminution in reproductive capacity over four generations. Experimentation on each generation was terminated after the birth of the first litters. From our observa-

tions on both the number of offspring and the testicular tissue of the male parents, it is apparent that throughout the reproductive period of the animals' life there was no evidence of sterility as a result of the daily ingestion of relatively large amounts of caffeine.

The discrepancy between our observations and those of Stieve ('30, '31) on the testicle of the rabbit and those of Vacca ('26) on the dog's testicle might conceivably be due to a species difference in response to caffeine. This explanation, however, can not be accepted without further experimentation. The experiments of Stieve, as well as those of Vacca, appear to have been poorly controlled. The only information available with regard to Stieve's animals is that they were pure blooded Russian rabbits, 7 to 16 months old with an average weight of 1865 gm for the females and 1887 gm for the males. In studies of this kind it is of paramount importance that environmental and nutritional conditions be the same for both the test and control animals before and during the experiment, for it has been shown that such factors as light (Fiske, '39, '41; Pomerat, '42) and vitamin deficiencies and inanition (Mason, '30, '33; Mason and Wolfe, '30; Truscott, '44) affect the pituitary which, as is well known, exercises through its gonadotropic hormone a direct action on the testicles. In Vacca's experiments ('26) not only were the four dogs used as test animals selected at random but also the amount of caffeine administered was obviously toxic. All the test animals became emaciated and had general tremors and convulsions.

The difference in our results as compared with those obtained by Hammill and Miller ('35) with respect to growth may be due to the fact that in their experiments the various groups of animals were not fed the same diet. The authors state that all the diets, the composition of which is not given, were satisfactory for experimental purposes, but it is possible that they might not have been equally effective in promoting growth.

This objection does not apply to the observations of Smith and Hambourger ('36). However, it should be noted that their animals were given 100 mg caffeine per kilo by stomach tube whereas ours ingested ad libitum the smaller amount of 40 to 50 mg per day. It is hoped that further experimentation now under way in our laboratory may show whether the differences between our results and theirs were due to the different amount of caffeine the animals received or the different experimental procedure.

SUMMARY

Albino rats were given a sweetened caffeine beverage as their sole source of fluid throughout their reproductive period.

The average daily consumption of caffeine by each animal amounted to 40–50 mg per kilo body weight.

The reproductive capacity as judged by the number of litters and number of offspring was not impaired by the daily ingestion of caffeine.

There were no pathological changes in the testicles of the test animals except for the natural histological degenerative changes which were observed in about equal frequency in the control animals.

The rate of growth of the animals on the caffeine beverage was the same as that of the controls.

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The Influence of Pregnancy on the Acute Toxicity of Various Compounds in Mice

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The Influence of Pregnancy on the Acute Toxicity of Various Compounds in Mice. BELILES, R. P. (1972). *Toxicol. Appl. Pharmacol.* **23**, 537–540. LD50 values were determined in pregnant (day 13) and nonpregnant mice. Iron dextran, iron dextrin, folic acid, colchicine and chlorpromazine hydrochloride were more toxic in the pregnant mouse than in the nonpregnant mouse following iv administration. Aspirin administered po appeared less toxic in the pregnant mouse. No remarkable differences between pregnant and nonpregnant animals in respect to the LD50 values were noted (a) following iv administration of atropine sulfate, caffeine, chlorpheniramine maleate, chloramphenicol, diphenhydramine hydrochloride, ethanol, ergonovine maleate, ferrous sulfate, hexobarbital sodium, isoproterenol hydrochloride, metyrapone ditartrate, pentobarbital sodium and trypan blue, and (b) following po administration of ferrous sulfate, folic acid and naladixic acid.

The Food and Drug Administration Advisory Committee on Protocols for Safety Evaluations (1970) indicated that the maternal organism was particularly vulnerable during pregnancy because of altered pathways of metabolism, variations in dietary intake, endocrine changes and the possibility of initiating spontaneous labor. No specific illustrations of altered toxicity were presented, although the biochemical data reviewed by Guarino *et al.* (1966) suggest that it is only logical to believe that such alterations do exist. Review of the literature did not suggest that this aspect of toxicity has been systematically considered with any materials.

In this investigation, acute toxicity was used as a criterion to evaluate possible changes in the sensitivity of female mice to various chemicals due to pregnancy.

METHOD

Pregnant adult female Charles River CD-1 mice mated at the Charles River Mouse Farms, Inc. were received by this laboratory. The day sperm or a vaginal plug was observed was considered day 0 of gestation. Nonpregnant adult female mice of the same age and strain were also supplied by The Charles River Mouse Farms, Inc. Upon receipt, the mice were group-housed in suspended wire-mesh cages and provided water and food (Rockland Mouse Breeder Diet) ad libitum.

On day 13 of gestation the pregnant mice were administered graded doses of test materials. A minimum of 4 dose levels and 5 mice per dose level were used. On day 17

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of gestation, a cesarean section was performed, and the contents of the uterus were inspected. The mice that died were also necropsied, and the pregnancy was confirmed. Implantation sites, resorption sites, dead and live fetuses were recorded. Any pregnant mouse in which total resorption of the litter was judged to have occurred before the day of compound administration was discarded and replaced with a suitable pregnant mouse. After the same length of time in our laboratory, nonpregnant mice received graded doses of the test materials. These mice were treated in a manner similar to the pregnant mice, except that nonpregnant mice were sacrificed after 7 days. Body weights at the time of treatment were recorded. All mice were observed daily. According to the method described by Horn (1956), the LD50 values were obtained in both pregnant and nonpregnant mice after 4 and 7 days, respectively. If the surviving pregnant females had resorbed or dead fetuses, the fetal LD50 value based on the dose given to the dam was calculated using the method of Litchfield and Wilcoxon (1949).

Ergonovine maleate,[1] chloramphenicol,[2] chlorpheniramine maleate,[3] diphenhydramine hydrochloride,[4] iron dextran,[5] iron dextrin[6] and metyrapone ditartrate[7] were obtained as injectable formulations, reconstituted as directed, if so required, and administered iv. Solutions of atropine sulfate, caffeine, folic acid, trypan blue, isoproterenol hydrochloride, hexobarbital sodium, ethanol, colchicine, ferrous sulfate, chlorpromazine hydrochloride and pentobarbital sodium were prepared using distilled water. Aspirin and naladixic acid[8] were suspended in 0.5% gum tragacanth.

RESULTS AND DISCUSSION

The LD50 values based on the mortalities observed in the 4 days after dosing and fetal LD50 values of compounds which produced sufficient fetal deaths to permit calculation have been tabulated in Table 1. The LD50 values obtained after 7 days observation period in the nonpregnant mice were identical except for folic acid (iv), iron dextran and iron dextrin. These values and 95% confidence limits were 282 (216–368) mg/kg for folic acid, 2050 (1510–2780) mg Fe/kg for iron dextran and 383 (308–476) mg Fe/kg for iron dextrin.

Iron dextran, iron dextrin, folic acid, colchicine and chlorpromazine HCl were more toxic in the pregnant mouse following iv injection. Aspirin administered po was less toxic. No remarkable differences were noticed between LD50 values following iv administration of atropine sulfate, caffeine, chlorpheniramine maleate, chloramphenicol, diphenhydramine HCl, ethanol, ergonovine maleate, ferrous sulfate, hexobarbital sodium, isoproterenol HCl, metyrapone ditartrate, pentobarbital sodium and trypan blue. There was no difference observed following po administration of ferrous sulfate, folic acid and naladixic acid. Fetal LD50 values suggest that only aspirin and chloramphenicol were significantly more toxic to the fetus than to the dam.

[1] Ergotrate maleate; Eli Lilly and Co., Indianapolis, Indiana.
[2] Chloromycetin sodium succinate; Parke-Davis and Co., Detroit, Michigan.
[3] Chlor-Trimeton; Schering Corp., Bloomfield, New Jersey.
[4] Benadryl; Parke-Davis and Co., Detroit, Michigan.
[5] Imferon; Lakeside Laboratories, Inc., Milwaukee, Wisconsin.
[6] Astrafer; Astra Pharmaceutical Products, Inc., Worchester, Massachusetts.
[7] Metopirone; Ciba Pharmaceutical Co., Summit, New Jersey.
[8] NegGram; Winthrop Laboratories, New York, New York.

TABLE 1

LD50 (95% CONFIDENCE LIMITS) VALUES (mg/kg) FOR NONPREGNANT, PREGNANT AND FETAL MICE AFTER ADMINISTRATION OF VARIOUS COMPOUNDS

Compound	Route	Nonpregnant	Pregnant[c]	Fetal[d]	LD50 ratio Nonpreg/Preg.
Aspirin	po	1040 (815–1320)	1400 (1080–1800)	760 (692–835)	0.74
Atropine sulfate	iv	96.3 (76.5–123)	82.6		1.16
Caffeine	iv	89.2 (68.4–116)	96.3 (79.8–116)		0.92
Colchicine	iv	4.13 (3.55–4.82)	1.54 (1.14–2.09)		2.68
Chlorpheniramine maleate	iv	26.1	26.1		1.25
Chlorpromazine HCl	iv	35.5 (30.4–41.3)	24.2 (19.0–30.8)		1.46
Chloramphenicol	iv	1530 (1260–1840)	1210	679 (643–716)	1.26
Diphenhydramine HCl	iv	35.5 (30.4–41.3)	28.2 (24.2–32.9)		1.25
Ethanol (50%)	iv	6.08[a] (5.22–7.08)	5.90 (4.38–7.94)		1.03
Ergonovine maleate	iv	8.26	8.26		1.00
Ferrous sulfate	po	1410 (1170–1700)	1310 (1120–1520)		1.03
Ferrous sulfate	iv	115 (81.0–164)	105 (74.1–148)	75.0 (64.8–87.0)	1.15
Folic acid	po	>10000	>10000		—
Folic acid	iv	305 (239–388)	178		1.71
Hexobarbital sodium	iv	165 (145–192)	178		0.92
Isoproterenol HCl	iv	96.3 (79.8–116)	89.2 (76.5–104)		1.08
Iron dextran	iv	2240[b] (1460–2700)	154 (109–218)		14.55
Iron dextrin	iv	446[b] (350–569)	127 (94.4–171)		3.51
Metyrapone ditartrate	iv	261	305 (252–368)		0.85
Naladixic acid	po	1120 (962–1310)	891 (699–1140)	658 (604–716)	1.25
Pentobarbital sodium	iv	112 (96.2–131)	121		0.92
Trypan blue	iv	328	261		1.25

[a] Values of ethanol (50%) given in ml/kg.
[b] Values given in terms of mg Fe/kg.
[c] Mice dosed on day 13 and examined by cesarean section on day 17 (4 day LD50).
[d] The fetal LD50 based on maternal dosage.

While the difference in LD50 values was greater with iron dextran, this material in terms of iron was the least toxic of all iron compounds that were administered iv. Additional acute studies in rats and rabbits have not revealed the marked effect of pregnancy on the acute toxicity of iron dextran in these species. For example, in female rats the LD50 values in the nonpregnant and pregnant rat obtained following iv administration on day 14 of gestation were both greater than 2000 mg Fe/kg.

During the course of this investigation the difference in body weights of pregnant and nonpregnant mice drew our attention. The mean body weight and standard deviation for the pregnant mice were 39.0 ± 3.5 g at the time of treatment. Analysis of the body weight data from the nonpregnant mice of the same age gave a mean value of 26.5 ± 2.8 g. Thus, the changes during conception caused an increase in body weight of approximately 12.5 g. This increase is composed in part, of the placenta, fetuses and increased size of the uterus. Based on the possibility that some differences in toxicity could be due to compartmentalization of the test material in the maternal organism, a theoretical upper limit of 1.40 was calculated for LD50 ratio (nonpregnant : pregnant) from the body weight data to account for this.

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The Effect of Caffeine on Free Fatty Acids

A Preliminary Report

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THE PHYSIOLOGIC effects of coffee are due entirely to its caffeine content. Following its oral administration it is rapidly absorbed and its effects are almost immediately apparent. Coffee is one of the most common beverages utilized in this and many other countries. The amount consumed by the average individual approximates three cups a day; many coffee drinkers exceed this figure, some consuming as many as ten to 15 cups a day. The average amount of caffeine contained in the usual percolator type of coffee is approximately 150 mg per cup, while "instant coffee" contains approximately 80 to 90 mg and decaffeinated coffee contains about 15 to 25 mg per cup.[1] Other beverages, such as cola drink,[2] contain from 46 to 53 mg per 12 ounce bottle.

The pharmacologic effects of caffeine are fairly well known; these include an increase in the excitability of the nervous system, in alertness, in basal metabolism, and in cardiac work; it has, in addition, a diuretic effect. Because caffeine induces central nervous system arousal and a generalized stimulating effect and thus constitutes a stress effect, it was considered of interest to study the effects of its administration on certain lipid fractions, particularly serum free fatty acids (FFA).

Materials and Methods

The effects of caffeine were studied following its administration in the form of coffee and as caffeine sodium benzoate administered intramuscularly.

Thirty-four normal male subjects (ages 18 to 63) were used in this study. All experiments were performed in the fasting state. The subjects were kept at rest and free from environmental stimulation and were prohibited from smoking during the period of the experiment. Statistical significance of the data obtained was determined by means of the *t*-test.

Studies With Coffee.—Two series of observations were made in 12 subjects. Five grams of soluble instant coffee powder (containing 218.0 mg of caffeine) dissolved in 500 ml of hot water, with three tablets of an artificial sweetening agent added (50 mg sodium cyclamate and 5 mg sodium saccharin/tablet), were ingested by each subject in a 15-minute period. This is the equivalent of 2½ cups of instant coffee. In the second series of experiments, 500 ml of hot water with three tablets of sweetening agent was given as a control.

Blood samples were taken from an antecubital vein before and 30, 60, 90, 120, and 180 minutes after the ingestion of the beverage. Serum free fatty acid (FFA) concentrations were determined in all experiments.[5] Serum triglycerides,[6] cholesterol,[6] and plasma glucose[6,7] were also determined.

Studies With Caffeine.—To determine the effect of caffeine on FFA, caffeine sodium benzoate (0.5 gm in buffered saline) was given intramuscularly to 11 subjects and blood samples taken for FFA determinations as above. As a control, sodium benzoate (0.5 gm in buffered saline) was given to 11 subjects and FFA curves obtained.

Results

Studies With Coffee.—Coffee Experiments: Free fatty acid concentrations after coffee ingestion are shown in Table 1. There was a rise in FFA in all subjects. The mean peak rise was 393±115 μEq/liter (67%). This was significant compared to the mean FFA response to the control beverage ($P < 0.05$). The maximal FFA elevation occurred at 180 minutes in each subject. It did not appear to be related to fasting FFA level or coffee-drinking habit. There was no significant change in serum cholesterol, serum triglycerides, or plasma glucose.

Control Experiments: The FFA response to the control beverage (water and sweet-

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TABLE 1.—*Free Fatty Acid Concentrations After Coffee and After Water in the Same Subject*

Subject	Beverage	Serum Free Fatty Acids (μEq/liter) Time (Minutes) 0	30	60	90	120	180
1	Coffee	340	329	392	623	733	790
	Water	406	330	376	388	294	541
2	Coffee	817	670	989	830	1232	1283
	Water	795	700	865	647	659	883
3	Coffee	736	518	600	670	989	1042
	Water	516	372	342	300	264	318
4	Coffee	387	419	406	374	329	516
	Water	502	514	453	563	790	606
5	Coffee	213	168	135	180	174	341
	Water	189	110	163	182	215	120
6	Coffee	536	529	750	964	854	1010
	Water	714	390	672	624	708	774
7	Coffee	266	357	340	554	617	744
	Water	564	582	258	306	366	300
8	Coffee	868	525	525	550	575	918
	Water	582	499	535	450	461	630
9	Coffee	825	581	931	712	812	932
	Water	324	318	257	214	183	159
10	Coffee	1068	1500	1156	1300	2462	2537
	Water	941	866	995	736	766	948
11	Coffee	249	402	644	714	881	884
	Water	164	—	394	477	541	647
12	Coffee	789	535	777	712	702	812
	Water	324	456	300	288	294	288

ener) was less than after coffee in 10 of the 12 subjects (Table 2). The mean increment was 99±46μEq/liter (20%). The maximal FFA elevation occurred at variable intervals after the beverage.

Studies With Caffeine.—Caffeine Experiments: Free fatty acid concentrations after intramuscular injections of caffeine sodium benzoate are seen in Table 2. There was a rise in FFA in all subjects.* The mean maximal rise was 210±46μEq/liter (46%). This was significant compared to the mean FFA response to injections of the control solution ($P<0.02$). The peak rise occurred at 120 minutes or earlier in the majority of experiments.

Control Experiments: The FFA response to injections of the control solution (sodium benzoate) was significantly less than after caffeine (Table 2). The mean rise was 58±30μEq/liter (13%). The maximal in-

* Similar increases in free fatty acid levels have been consistently observed by us in experiments in dogs.[2]

TABLE 2.—*Free Fatty Acid Concentrations After Intramuscular Injections of Caffeine Sodium Benzoate and Sodium Benzoate*

Caffeine Sodium Benzoate: Serum Free Fatty Acids (μEq/Liter) Time (Minutes)						Sodium Benzoate: Serum Free Fatty Acids (μEq/liter) Time (Minutes)					
Subject	0	30	90	120	180	Subject	0	30	90	120	180
13	260	349	377	349	374	24	325	335	255	344	290
14	335	307	505	545	535	25	251	267	202	182	162
15	323	336	305	388	417	26	326	328	305	341	331
16	590	515	755	690	740	27	545	590	615	585	535
17	458	491	481	745	665	28	1045	1205	1145	1300	1336
18	384	570	645	675	1015	29	497	319	232	400	287
19	505	675	500	550	780	30	333	—	397	334	395
20	625	571	680	595	795	31	523	538	549	673	609
21	625	655	755	810	660	32	300	326	300	331	378
22	675	670	750	785	840	33	425	431	367	390	478
23	203	225	246	265	249	34	437	408	274	392	344

TABLE 3.—*Free Fatty Acid Elevation After Coffee Ingestion and Intramuscular Caffeine*

	No. of Subjects	Initial Mean FFA Concentration (μEq/liter)	Maximal FFA Increment Mean ± SE (μEq/liter)	%	P*
Coffee	12	591	393±115	67	<0.05
Control (water)	12	505	99± 46	20	
Caffeine Sodium Benzoate	11	453	210± 46	46	<0.02
Control (Sodium Benzoate)	11	455	58± 30	13	

* Significance of difference between means of test group and corresponding control group.

crement occurred at varied times after injection.

Comment

The experiments showed a consistent and significant effect of ingested coffee and injected caffeine on FFA mobilization. There was a four-fold greater response than that which followed a control beverage and control injection (Table 3). The similarity in FFA effect in the coffee and caffeine experiments did not appear to be influenced by the use of the same individual as test and control subject in the coffee group but not in the caffeine group. The similar FFA response to coffee and caffeine and their respective control solutions suggests that the caffeine in the coffee was responsible for the FFA rise which occurred. The earlier rise of FFA concentrations after intramuscular caffeine was probably due to the route of administration.

The mechanism of the FFA response to coffee is not clearly understood. Based on our own observations[2] and on the work of others,[10] it does not appear to be due to simple FFA mobilization due to catecholamine release. The observed effect of caffeine on FFA is at the present time under investigation. It has been shown that an increase in plasma FFA may predispose to a rise in lipoprotein lipids.[8] A recent epidemiologic study has shown a positive association between coffee intake and coronary heart disease.[9] The FFA effect after caffeine observed in the present study may be related to this clinical observation.

Summary

The effect of caffeine in the form of caffeine sodium benzoate and coffee on serum free fatty acids (FFA) was studied in a group of human subjects. The FFA were found to be significantly elevated after coffee. Significant elevation in FFA was similarly observed after caffeine sodium benzoate administered intramuscularly. These findings are of interest in view of the suggested relationship of FFA elevation to that of other lipid fractions, notably triglycerides and cholesterol.

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Response of Free Fatty Acids to Coffee and Caffeine

By Samuel Bellet, Alfred Kershbaum and E. Michael Finck

The effect of coffee, decaffeinated coffee and a control beverage on plasma FFA was studied in a group of normal human subjects. The effect of caffeine sodium benzoate was also studied in the dog. Significant elevations in FFA were observed after coffee and caffeine which usually reached their peak in 3 or 4 hours. The administration of sucrose significantly reduced the immediate FFA response. The FFA effects with decaffeinated coffee were markedly less than with regular coffee and were similar to that of the control beverage. These effects are considered to be of importance, particularly in that they may be related to other disturbances in lipid metabolism. (Metabolism 17: No. 8, August, 702-707, 1968)

COFFEE is one of the most common beverages utilized in this and in many other countries. The amount consumed by the average individual approximates three cups a day; many coffee drinkers exceed this figure, some consuming as many as 10 to 15 cups a day. The physiologic effects of coffee are due entirely to its caffeine content.[1-3] Following its oral administration, it is rapidly absorbed and its effects are almost immediately apparent. The average amount of caffeine content contained in the usual percolator type of coffee is approximately 150 mg./cup, while "instant coffee" contains approximately 80 to 90 mg. and decaffeinated coffee contains about 15 to 25 mg./cup.[4]

The pharmacologic effects of caffeine are fairly well known; these include an increase in the excitability of the nervous system, in alertness, in basal metabolism, and in cardiac work; it has, in addition, a diuretic effect. Through its excitatory effects on the central nervous system, caffeine produces a stress effect.

There is little data available pertaining to the relationship of caffeine to certain lipid fractions. The purpose of this communication is to report our experience relative to the effect of caffeine and caffeinated beverages on plasma concentrations of free fatty acids (FFA) in man and in the dog.

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Table 1.—*Plasma Free Fatty Acid Concentrations (μEQ/L) in 10 Men After Ingestion of Coffee, Decaffeinated Coffee, Water and Coffee plus Sucrose*

Hours	−1	0	1	2	3	4
Coffee	497* ±45	432 ±47	614 ±55	677 ±51	816 ±89	831 ±67
Decaffeinated Coffee	534 ±42	512 ±50	496 ±68	530 ±48	605 ±32	637 ±72
Water	425 ±52	400 ±40	381 ±32	341 ±37	417 ±44	404 ±44
Coffee plus Sucrose	396 ±29	373 ±28	277 ±23	283 ±26	493 ±50	638 ±63

*Mean ± S.E.

Materials and Methods

Human Subjects

The effects of caffeine were studied following its administration in the form of coffee and decaffeinated coffee. The subjects were normal, male volunteers, ages 21 to 39 (average age, 29 years). Their health status was determined from the history, physical examination, and routine laboratory studies. These subjects regularly drank 2 to 6 cups of regular instant coffee per day with an average intake of 3 to 4 cups. Only subjects who presented no abnormalities were included in this study. All experiments were performed in the fasting state. The subjects were kept at rest and free from environmental stimulation and were prohibited from eating or smoking during the period of the experiment and for 8 hours prior to the study. The following studies were performed in randomized sequence at weekly intervals in 10 subjects (Group A, B, and C): (A) 5 Gm. soluble instant coffee powder (containing 250 mg. caffeine) dissolved in 500 ml. hot water, with 3 tablets of an artificial sweetening agent added (50 mg. sodium cyclamate and 5 mg. sodium saccharin/tablet) were ingested by each subject during a 15-minute period. This is equivalent to 2½ cups of instant coffee. (B) The administration of 5 Gm. of decaffeinated coffee (containing 20 mg. caffeine) dissolved in 500 ml. hot water with 3 tablets of the same artificial sweetener. (C) 500 ml. hot water with 3 tablets of sweetening agent was given as a control. (D) The administration of 5 Gm. of instant coffee as in (A) (containing 250 mg. of caffeine) plus 25 Gm. of sugar (sucrose).

Blood samples were taken from an antecubital vein before and one, two, three, and four hours after coffee ingestion. Plasma free fatty acid concentrations were determined in all subjects;[5] plasma caffeine concentrations[1] were also determined in many of the subjects. Data were analyzed for statistical significance by Student's t-test.

Animal Studies

Twenty-six mongrel dogs of both sexes, weighing 7 to 18 Kg., were anesthetized with sodium pentobarbital (25 mg./Kg.). Intramuscular caffeine (caffeine sodium benzoate, 25 mg./Kg. of caffeine) was given to 13 of the animals and 13 were used as controls.

Venous blood samples were collected from a femoral vein at 30, 20, 10, and 0 minutes prior to the intramuscular injection of caffeine and at 30, 60, 120, 180, and 240 minutes after caffeine. A similar procedure was followed in the control animals, without the administration of caffeine.

Results

Studies in Human Subjects

Effect of coffee in plasma FFA concentrations. The results of these experiments are shown in Table 1. There was a rise in FFA in all subjects, the peak concentration usually occurring 3 or 4 hours after coffee ingestion. The mean

Table 2.—*Mean Changes in Plasma Free Fatty Acids (μEQ/L) After Coffee, Decaffeinated Coffee, and Water*

Hours	0	1	2	3	4
Coffee	0	+182*	+245*	+384*	+399*
Decaffeinated Coffee	0	− 16†	+ 18†	+ 93†	+125†
Water	0	− 19	− 59	+ 17	+ 4

*Significant compared to decaffeinated coffee and water (P<0.001).
†Not significant compared to water.

increase in FFA concentration was 92 per cent above the initial pre-coffee level. The FFA response to coffee was markedly greater than that following decaffeinated coffee or water (Table 2).

Effect of decaffeinated coffee. Plasma FFA concentrations showed a moderate increase after decaffeinated coffee, with a mean rise of 24 per cent above the initial concentration (Table 1). The response was significantly less than that which followed regular coffee. Compared to the water control beverage, the FFA rise after decaffeinated coffee was greater at 2, 3, and 4 hours, but the differences were not statistically significant (Table 2).

Effect of water control. There was no appreciable change in mean FFA concentrations after the control water beverage (Table 1).

Effect of coffee plus sucrose. There was a suppression of the FFA response to coffee in all subjects when the experiment was repeated with coffee containing sucrose (Table 1). After the coffee-sucrose beverage, the mean FFA concentration dropped below the initial value for the first 2 hours and then rose to a peak level at 4 hours. Compared to coffee without sucrose the FFA response was significantly less for the first 3 hours. At 4 hours the FFA rise after coffee plus sucrose was somewhat less than the rise after plain coffee, but the difference was not statistically significant (Table 3).

Effect of coffee on blood caffeine concentration. Following the administration of caffeine in the form of coffee, it has been shown that the concentration in the blood reaches its maximum within one hour, after which it gradually declines over a period of 7–9 hours.[1] In our experiments, the caffeine concentration was determined following the administration of coffee (5 subjects), after decaffeinated coffee (7 subjects), and in control subjects given water (5 subjects). The averages of the caffeine concentration are given in Table 4. Appreciable plasma concentrations were observed after coffee; these were markedly less after decaffeinated coffee and the control beverage.

Table 3.—*Mean Changes in Plasma Free Fatty Acids (μEQ/L) After Coffee, and Coffee plus Sucrose*

Hours	0	1	2	3	4
Coffee	0	+182*	+245*	+384*	+399†
Coffee plus Sucrose	0	− 96	− 90	+120	+265

*Significant compared to coffee plus sucrose (P<0.001).
†Not significant.

Table 4.—*Plasma Caffeine (μg/100 ml.) Concentrations After Coffee, Decaffeinated Coffee, and Water*

Beverage	Number of Subjects	0	1 hr.	2 hrs.	3 hrs.	4 hrs.
Coffee	5	0.962	5.974	3.670	2.535	2.742
Decaffeinated Coffee	7	0.670	0.866	1.147	1.119	0.419
Control (Water)	5	0.597	0.678	0.638	1.750	0.990

Following the administration of sucrose with coffee as in Experiment D, the plasma concentrations were similar to those when coffee alone was given. The difference in FFA response therefore is probably due to the difference in FFA mobilization from adipose tissue.

Animal Studies

Caffeine increased the mean serum FFA level 137 per cent above the pre-coffee value (Fig. 1). Compared to FFA changes in anesthetized control dogs, this was highly significant ($P<0.01$). The peak FFA concentration occurred 180 minutes after caffeine.

Discussion

The above studies demonstrated significant and consistent effects of ingested coffee and injected caffeine on FFA mobilization. The response was markedly greater than that which followed decaffeinated coffee or a control beverage.

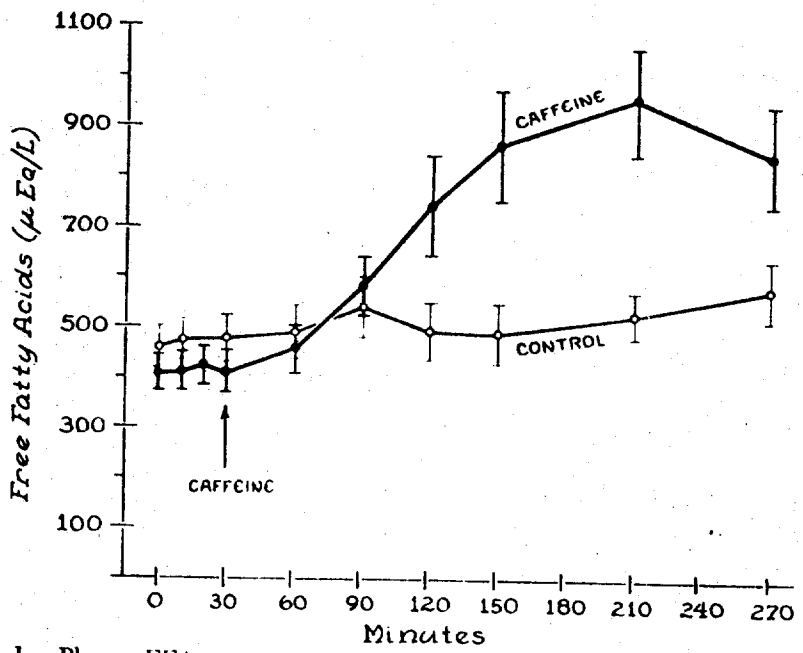

Fig. 1.—Plasma FFA concentrations in 13 anesthetized dogs after caffeine sodium benzoate (25 mg. Kg.) administered intravenously. Note that rise begins within 30 minutes after its administration. Peak is attained at about 3 hours; then FFA concentration gradually decreases.

The similarity in FFA effect of coffee and caffeine administered in similar dosage has been previously noted by us.[2] The plasma concentration of caffeine given in the form of coffee or intramuscular caffeine sodium benzoate has been shown to be identical.[1]

Effect of sucrose. Sucrose decreased the initial FFA response to coffee. The diminished response of FFA to coffee containing sucrose is probably due to the suppression of FFA release by the sucrose as a result of its effect on re-esterification of the released FFA.* This later was reversed by the lipolytic action of caffeine and at 4 hours the FFA increment was not significantly less than that which followed coffee without sucrose.

Mechanism of effect. The xanthines manifest a pronounced effect on adipose tissue metabolism. Sutherland and associates[11,12] have shown that cyclic 3′,5′-AMP promotes lipolysis in adipose tissue. The enzyme, phosphodiesterase, which antagonizes the activity of cyclic 3′,5′-AMP, is inhibited by caffeine and other xanthines. As a result, the concentration of 3′,5′-AMP in adipose tissue rises and an enhanced lipolysis results. Catecholamines also increase the concentration of cyclic 3′,5′-AMP in adipose tissue but by a different mechanism. The cyclic 3′,5′-AMP increases cellular lipase and lipolysis and is therefore also the apparent mediator of catecholamine lipolysis.[11,12]

Studies so far indicate that caffeine also has an effect on catecholamine release. There was a significant increase in urinary catecholamine excretion after coffee in 4-hour short-term studies.[13] This would suggest that the FFA response to caffeine is also the result of catecholamine lipolysis. The persistence of FFA mobilization after blocking agents and adrenalectomy in dogs supports this dual action.[13] The mechanism of caffeine lipolysis differs in this respect from the FFA mobilization induced by nicotine, which results from sympathoadrenal stimulation.[14] The FFA elevation after coffee is greater and more prolonged than after tobacco smoking.[15]

Significance of these studies. The observed effect of caffeine on FFA is of considerable importance as related to lipoprotein metabolism. It has been shown that an increase in plasma FFA predisposes to a rise in lipoprotein lipids.[16] Recently, evidence of a direct relationship of plasma FFA concentration to lipoprotein synthesis by the liver was reported.[17] In addition, several lines of evidence support the conclusion "that elevated plasma levels of FFA, and the consequent elevated rate of uptake of FFA by the liver, lead in a rather direct manner to an increase in the rate of production and secretion of lipoproteins into the plasma."[18]

A recent epidemiological study has shown a relation between caffeine intake and coronary heart disease.[19] It has also been reported that significant correlations between coffee drinking and serum cholesterol and lipoprotein concentrations were found in men with coronary heart disease but not in healthy controls.[20]

The findings observed in this study are of interest because of their possible relationship to other disturbances in lipid metabolism.

*Increase in FFA produced by various agents is suppressed by the administration of glucose or sucrose.[6-10]

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Effect of Cola Drinks on Serum Free Fatty Acids

Manuel Bellet, MD; Alfred Kershbaum, MD; and Laurian Roman, MD, Philadelphia

The effect of cola drinks on free fatty acids (FFA) is of considerable interest. Diet cola drinks which contain caffeine and artificial sweetener but no sucrose produced significant progressive increase in plasma FFA during the first four hours after ingestion. The regular cola drinks which contain large amounts of sucrose in addition to the caffeine resulted in a decrease in the plasma FFA during the first two to three hours after ingestion, following which there was an increase by the fourth hour. The diminished response of FFA to the cola drinks containing sucrose is probably due to the suppression of caffeine-induced FFA release by the sucrose as a result of its effect on the resesterification of the released FFA. These findings are of interest in view of the relationship of FFA elevation and other lipid fractions, notably, triglycerides and cholesterol.

THE COLA drinks are among the most extensively used beverages in this country; the beverage is imbibed by children as well as by adults. The cola drinks appear in two forms: the regular cola beverages, which contain sugar in the form of sucrose; and the "diet" or sugar-free colas, which employ a sugar substitute (cyclamate sodium or calcium). The beverages contain primarily caffeine, sugar (except in the diet cola), and carbonated water; other contents include sodium, potassium, and phosphoric acid.

In previous publications, we have reported on the effects of coffee and caffeine on

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Table 1.—*Plasma FFA Concentrations (μEq/liter) After Cola, Sugar-Free Cola, and Carbonated Water Ingestion**

	Cola Beverage					Sugar-Free Cola Beverage					Carbonated Water (Control)				
Subject	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
1	811	698	519	491	1,164	457	833	947	796	1,004	409	518	541	382	316
2	454	418	385	487	694	562	389	668	711	420	582	605	605	668	790
3	600	515	471	596	645	351	466	452	360	567	409	326	764	311	528
4	704	382	347	600	898	466	367	475	622	868	567	555	601	747	606
5	456	456	555	1,011	897	392	579	773	855	963	493	642	439	457	398
6	580	262	570	639	823	743	734	760	803	1,335	419	414	278	363	493
7	442	510	442	370	452	340	525	592	600	631	555	634	742	770	742
8	492	442	420	524	618	362	442	415	578	700	504	504	612	578	651
9	631	414	295	512	719	418	616	569	647	710	539	611	689	511	681
10	647	611	440	409	602	668	726	668	590	632	694	556	439	706	706
11	476	507	254	265	906	559	480	611	538	465	761	750	678	484	478
12	880	404	420	663	600	544	342	513	507	409	395	400	450	611	467

*Values are given for 0, 1, 2, 3, and 4 hours after ingestion.

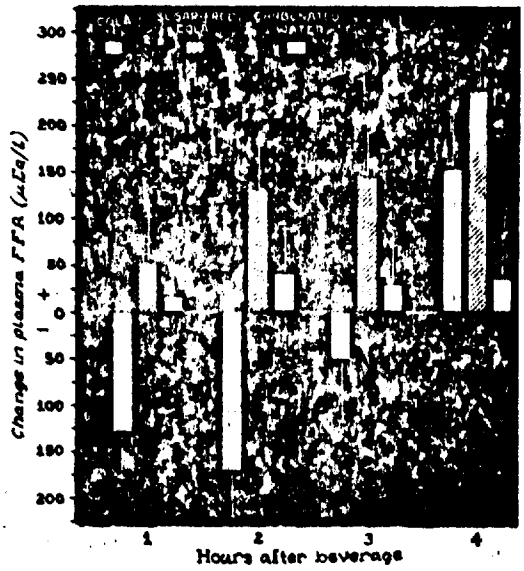
Table 2.—*Change in Plasma FFA Concentrations After Cola, Sugar-Free Cola, and Carbonated Water Ingestion*

Beverage	Initial Mean FFA Concentration (μEq/liter)	Change in FFA Concentration (mean ± SE) at 4 hr (μEq/liter)
Cola*	598	+ 155 ± 61
Sugar-free cola*	489	+ 237 ± 80
Carbonated water†	527	+ 41 ± 40

*Mean differences between initial levels of FFA and levels at 4 hr after ingestion of regular cola or diet cola are both statistically significant ($P < 0.05$).
†Mean difference between initial FFA levels and levels at 4 hr after ingestion of carbonated water are not statistically significant.

free fatty acid (FFA) metabolism in human subjects and in the experimental animal.[1-3] The purpose of this communication is to report our experience concerning the effect of various cola drinks on FFA in normal subjects.

Time course of changes in plasma FFA concentrations (mean and standard error) in 12 normal male subjects after cola, sugar-free cola, and carbonated water ingestion.



Methods and Materials

The effect of cola drinks was studied in 12 healthy, male subjects, aged 14 to 42 years. Three different studies were performed in each subject: (1) ingestion of 16 oz regular cola; (2) ingestion of 16 oz sugar-free cola (diet cola); and (3) ingestion of 16 oz carbonated water. Subjects participated in only one experiment per day in random order, and the interval between experiments was at least five days. All studies were performed at 9 AM, with no food having been taken since the previous evening meal. Subjects were kept at rest and free of environmental stimulation and no smoking was permitted during the night preceding the study or during the period of the experiment. Blood samples were taken from an antecubital vein before and one, two, three, and four hours after beverage ingestion. Plasma FFA concentrations were determined by the method of Gordon.[4]

The caffeine content of various brands ranges from 3.0 to 7.0 mg per fluid ounce.[5,6] Therefore, the average caffeine content of a 16-oz bottle of cola is close to that of a cup of coffee (approximately 100 mg). The sugar-free colas generally contain lesser amounts of caffeine

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3	600	515	471	596	645	351	466	452	360	567	409	326	764	311
4	704	382	347	600	898	466	367	475	622	868	567	555	601	747
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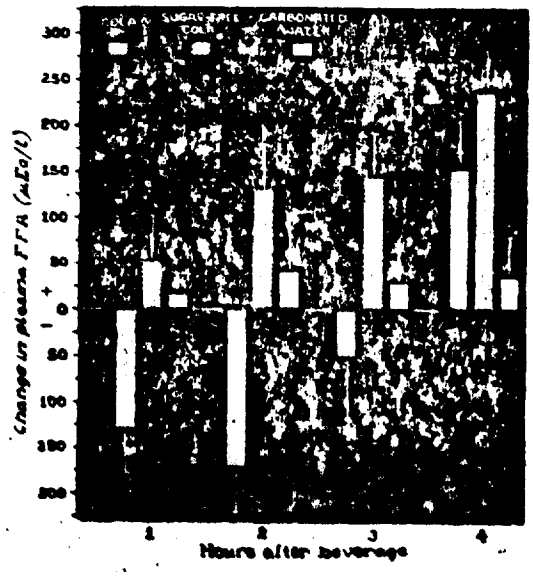

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than the regular colas. The sugar content of the regular colas is approximately 30 to 55 gm per 16-oz. bottle.[5]

Our own laboratory estimates of the caffeine content in various colas were as follows: regular cola, 6.0 to 7.0 mg/oz; and sugar-free cola, 3.2 to 3.5 mg/oz. Caffeine and sucrose contents of the cola beverages employed in this study were as follows: regular cola, 109 mg caffeine and 35 gm sucrose/16-oz bottle; sugar-free cola, 46 mg caffeine/16-oz bottle. The control beverage consisted of carbonated water, no caffeine, and no sucrose.

Results

Plasma FFA concentrations after cola, sugar-free cola, and carbonated water ingestion are shown in Table 1. The mean ranges in FFA concentration at four hours after beverage ingestion are seen in Table 2. The Figure shows the time course of mean FFA change.

The mean FFA concentration after sugar-free cola rose steadily to reach a maximal level at four hours (48% above initial concentration). This increase is statistically significant ($P < 0.05$).

There was no appreciable change in the mean concentration after carbonated water ingestion.

After regular cola ingestion, there was a fall in mean FFA concentration which reached a nadir at two hours. This was followed by an elevation which reached its maximum at four hours. Compared to the index values, this increase is statistically significant ($P < 0.05$).

Comment

As in other caffeinated beverages, the physiological effects of cola depend upon the caffeine and sugar content. The pharmacologic effects of caffeine are fairly well known; these include increases in the excitability of the nervous system, in alertness, in basal metabolism, and in cardiac work. It has, in addition, a diuretic effect. Unlike coffee, the regular cola drinks contain considerable amounts of sugar already incorporated into the product; whereas coffee may be consumed with or without sugar. In sugar-free colas the sugar is replaced by a sugar substitute.

The Effects of Cola on Plasma FFA.—These studies show that ingestion of cola drinks containing caffeine (sugar-free cola) alone results in a progressive increase in FFA; when the cola contained caffeine plus sugar (regular cola), the FFA usually became lower in the first three hours and then tended to increase by the fourth hour after ingestion. These effects were quite similar to those obtained following the administration of coffee alone and coffee with sugar, respectively.[3] The ingestion of carbonated water had no significant effect on FFA.

Mechanism of Effects.—Caffeine, as do other xanthines, manifests a pronounced effect on adipose tissue metabolism. It has been shown that caffeine produces lipolysis in adipose tissue. Sutherland and associates[7,8] have shown that cyclic 3′, 5′ cyclic adenosine monophosphate (AMP) produces lipolysis in adipose tissue. The enzyme phosphodiesterase, which restricts production of cyclic 3′, 5′-AMP, is inhibited by caffeine and other xanthines. As a result, the concentration of 3′, 5′-AMP in adipose tissue rises and an enhanced lipolysis results. In addition, caffeine and other xanthines produce an increase in catecholamine secretion (Bellet, S.: The Effect of Coffee Ingestion on Catecholamine Release, unpublished data).[9-11] Thus, the increase in plasma FFA could result from the increase in cyclic 3′, 5′-AMP due to inhibition of phosphodiesterase as well as the increase in catecholamine secretion.

Influence of Sucrose.—It is of interest that the sugar-free cola which contains caffeine but no sucrose showed an increase in FFA similar to that following coffee ingestion and intramuscular administration of caffeine. However, after ingestion of regular cola which contained a relatively high content of sucrose (35 gm/16-oz bottle), there was a decrease in FFA during the first three hours. The diminished response of FFA to cola beverage containing sugar was probably due to the suppression of caffeine-induced FFA mobilization by the sucrose as a result of its effect of reesterification of the released FFA. The effect of glucose on FFA metabolism has been well documented.[12-18] After glucose is given, a fall in plasma FFA concentration is noted.[18,19] When the effect of glucose administration has dissipated, the lipolytic effect of caffeine is no longer com-

teracted by the glucose and a significant increase in plasma FFA is observed.

Significance of FFA Response.—The effect of caffeine on FFA may be of considerable importance. It has been shown that an increase in plasma FFA predisposes to a rise in lipoprotein lipids.[20] A recent epidemiological study[21] revealed a positive relationship between coffee intake and coronary heart disease. Recent studies of patients with coronary artery disease[22] have shown a significant correlation between coffee drinking and increased serum cholesterol and lipoprotein levels. Recently, evidence has been obtained which leads to the conclusion that elevated plasma levels of FFA and the consequent uptake of FFA by the liver lead to an increase of lipoproteins in the plasma.[23] These findings indicate that caffeinated beverages, including cola drinks, which are used extensively by children as well as adults, merit further study from the standpoint of possible effects on the blood lipid fractions.

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Effect of Coffee Ingestion on Adrenocortical Secretion in Young Men and Dogs

By SAMUEL BELLET, JOHN KOSTIS, LAURIAN ROMAN AND OSCAR DECASTRO

Studies performed in 13 young, normal, healthy subjects have shown that ingestion of moderate amounts of coffee results in a significant increase in urinary 11-OHCS which reflects a stimulation of the adrenocortical activity. This conclusion is supported by our experiments in dogs in which we found a significant increase in the plasma 11-OHCS level in the phrenico-abdominal vein after administration of caffeine. These findings in addition to the observations that caffeine results in significant elevations in FFA and catecholamines, are of particular interest in view of the possible relationship of these changes to an increase in certain lipid fractions, particularly triglycerides and cholesterol. (Metabolism 18: No. 12, December, 1007-1012, 1969)

COFFEE AND OTHER CAFFEINATED BEVERAGES are widely used and many of the pharmacologic effects of caffeine are fairly well known. However, there are many aspects of its action which are of interest but have received little attention in the past. Studies from this laboratory[1-3] have shown an increase in free fatty acids and in catecholamine excretion following the ingestion of coffee, but as far as we are aware, very little work has been performed relative to the effect of caffeine on adrenocortical secretion. Such data would be of considerable clinical and theoretical importance from many standpoints, particularly because of its relation to the stress mechanism and various lipid fractions. The object of this study was to investigate the effect of caffeine on the adrenocortical activity. Our studies were performed in human subjects and in dogs.

METHODS AND MATERIALS

Studies Performed in Human Subjects

Thirteen normal, young male subjects, aged 18–22 years, were included in this study. None were on any drug therapy. During the night prior to the experiment, they were instructed not to smoke, eat or drink; at 7:30 a.m. when arising, after voiding they drank two glasses of water. All subjects reported to the laboratory at 8:30 a.m. They rested until

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9:00 a.m. when they voided and immediately thereafter the experiments were started. In each subject, the experiment consisted of the ingestion of 5 Gm. instant coffee (220 mg. caffeine) which is equivalent to 2 cups of coffee, dissolved in 500 ml. water and a control study consisting in the ingestion of 500 ml. water. The sequence of these control and caffeine experiments was randomized. Between the two studies, there was a free interval of at least five days. During both studies, the subjects rested in an armchair from 9:00 a.m. until 12:00 noon. Urine voided during this period was collected. The subjects voided at the end of the three-hour period so that the entire amount of urine secreted during the three-hour period of the experiment was collected. During the experiments, the subjects were kept isolated, only one in a room, in relaxed surroundings, with no smoking, eating, or drinking. The urine collected was analyzed for creatinine and for levels of 11-hydroxycorticosteroid (11-OHCS) by the fluorometric technique of Mattingly.[4] The 11-OHCS include cortisol, 20-hydroxycortisol and corticosterone.

Studies in Experimental Animals

Eight mongrel dogs (5 male and 3 female) weighing between 35 and 55 Kg. were anesthetized with sodium pentobarbital, intravenously, in the dose of 25 mg./Kg. The trachea was intubated and ventilation was maintained by the use of a standard Harvard Respirator. The femoral vein was exposed via surgical cutdown and a Number 7 KIFA specially shaped catheter was introduced and advanced to the inferior vena cava. The catheter was then guided into the left renal vein where injection of 20–30 cc. of 76 per cent Renografin was performed. The catheter was then withdrawn to the inferior vena cava and introduced into the vein (left phrenico-abdominal) immediately cephalad to the renal vein. This vein is usually situated at the level of the second lumbar vertebral body. In the dog the left adrenal vein drains into the left phrenico-abdominal vein. Although some mixture occurs, the latter reflects, in good measure, the changes in 11-OHCS concentration in the adrenal vein.

The nephrographic and pyelographic effect resulting from the injection of Renografin was employed to identify the vessels and localize the kidney. The position of the catheter in the left phrenico-abdominal vein was ascertained by the injection of 10 cc. of Renografin. A continuous drip of 5 per cent glucose in water containing Heparin (10 mg./1000 cc.) was started to maintain patency of the catheter.

Blood specimens from the phrenico-abdominal vein were obtained five minutes before and 15, 30 and 60 minutes after the intravenous administration of caffeine sodium benzoate in a dose of 50 mg./Kg. Control experiments were performed in the same dogs by injecting the same dose of sodium benzoate alone. The sequence of these caffeine and control studies was randomized.

RESULTS

Our findings in human subjects are shown in Tables 1 and 2. As can be seen from Table 1, the urinary 11-OHCS excreted during a period of three hours following the ingestion of coffee was higher than during a comparable control period in 12 out of 13 subjects studied. The statistical evaluation of our results is given in Table 2. A paired comparison of the data reveals a mean difference of 21.8 μg. in the urinary 11-OHCS between the coffee and the control studies. The standard error of the mean is equal to 3.70. This difference was highly significant

Table 1.—Urinary 11-OHCS Excretion in Human Subjects in Control Study and After Coffee Ingestion (μg./3 hr.)

Subject	1	2	3	4	5	6	7	8	9	10	11	12	13
Control	20	21	12	82	74	38	36	50	45	46	34	33	12
Coffee	52	52	32	110	120	50	67	80	50	59	61	44	10

Table 2.—Statistical Evaluation of Results in Human Subjects

Subject	Mean Excretion During 3 hrs. After Coffee Ingestion (μg.)	Mean Excretion During 3 hrs. After Water Ingestion (μg.)	Mean Difference	Variance	Standard Error of Mean	t	p
13	60.54	38.73	21.81	177.98	3.70	5.89	< 0.001

($p < 0.001$). The urinary creatinine excretion was not significantly different between the coffee and control experiments.

The results obtained in dogs are shown in Tables 3 to 5. As can be seen in Table 3, in each dog the 11-OHCS levels in the phrenico-abdominal vein were increased after administration of caffeine, whereas in the control studies with sodium benzoate alone no increase was noted. The peak concentration occurred at about 30 minutes in most animals. The statistical evaluation is given in Tables 4 and 5. As can be seen from these tables, the mean concentration of 11-OHCS was 11.7 μg./100 ml. before the administration of the drug; 15 minutes after its administration, the mean concentration was 36.9 μg.; at 30 minutes, 62.6 μg.; and at 60 minutes, 44.6 μg. When the data were analyzed by the student's t test for paired data, the difference between the 11-OHCS concentration before and 30 minutes after the injection of caffeine is significant with $p < 0.02$. The difference between the samples drawn before and at 15 minutes and 60 minutes after administration of caffeine were not significant (Table 4). The difference between the 11-OHCS concentration 30 minutes after the injection of caffeine and 30 minutes after the administration of sodium benzoate is significant with $p < 0.02$. The differences at 15 and 60 minutes after the injection of caffeine or sodium benzoate are also significant with $p < 0.05$ (Table 5). It appears that intravenous caffeine administration significantly increases the concentration of 11-OHCS in the phrenico-abdominal venous blood. This increase is most marked 30 minutes after the injection and starts declining after 60 minutes.

Table 3.—11-OHCS Levels in Phrenico-Abdominal Venous Blood in Dogs (μg./100 ml.)

Dog	Experiment	Baseline	15 min.	30 min.	60 min.
1	Control	12	9	6	9
	Caffeine	10	93	61	25
2	Control	12	10	12	8
	Caffeine	7	8	11	21
3	Control	16	11	11	16
	Caffeine	33	31	51	29
4	Control	9	6	6	7
	Caffeine	6	83	81	97
5	Control	5	3	4	5
	Caffeine	8	17	33	24
6	Control	13	10	10	20
	Caffeine	15	29	141	116
7	Control	9	8	9	10
	Caffeine	10	24	116	38
8	Control	5	4	3	4
	Caffeine	5	10	7	7

Table 4.—Statistical Evaluation of Results in Dogs, 11-OHCS Levels in Phrenico-Abdominal Vein Before and After Administration of Caffeine Sodium Benzoate (μg./100 ml.)

Minutes After Administration of Caffeine	Number of Dogs	Mean 11-OHCS Levels		Mean Difference	Variance	Standard Error of Mean	t	p
		Before Caffeine	After Caffeine					
15	8	11.7	36.87	25.1	1181.5	12.15	2.066	N.S.
30	8	11.7	62.63	50.8	2228.7	16.69	3.046	< 0.02
60	8	11.7	44.63	32.9	1616.7	14.21	2.314	N.S.

Table 5.—Comparison of 11-OHCS Levels in Phrenico-Abdominal Vein at Serial Time Intervals After Administration of Caffeine Sodium Benzoate and After Administration of Sodium Benzoate (Control) (μg./100 ml.)

Minutes After Administration of Caffeine	Number of Dogs	Mean 11-OHCS Levels		Mean Difference	Variance	Standard Error of Mean	t	p
		Control (4 cc.)	Caffeine-treated (4 cc.)					
0	8	10.1	11.7	1.6				N.S.
15	8	7.6	36.9	29.3	1320.365	11.5	2.543	< 0.05
30	8	7.6	62.6	55.0	2216.857	16.65	3.303	< 0.02
60	8	9.9	44.6	34.75	1343.429	12.96	2.681	< 0.05

Discussion

Our results indicate that in young, normal subjects the ingestion of a relatively moderate amount of coffee markedly enhances the 11-OHCS urinary excretion; this increase is statistically highly significant. The urinary excretion of 11-OHCS is considered to be an accurate index of the release by the adrenal cortex of these hormones.*

The fact that caffeine stimulates adrenocortical secretion is shown also by the experiments in dogs. In these experiments we found a statistically significant increase in the plasma 11-OHCS in the phrenico-abdominal vein (into which the blood from the adrenals drain) after administration of caffeine.

The mechanisms by which caffeine results in adrenocortical stimulation is at the present time under study. In previous work it has been found by us[3] and others[8-10] that caffeine manifests a significant stimulating effect on catecholamine release. It has been shown that administration of epinephrine results in an increase in ACTH release and in plasma 11-OHCS levels.[11] Therefore, a possible mechanism by which caffeine increases the 11-OHCS secretion is by stimulation of catecholamine release which in turn enhances the corticosteroid secretion.

Coffee and caffeinated beverages are today widely used in this and in many other countries, but surprisingly little attention has been focused on the many effects of their prolonged use, particularly in relation to their stress effects.

Several studies have shown that increased adrenocorticosteroid activity leads

* In this study we have used a method recently introduced by Mattingly in which the free plasma or urinary 11-OHCS is measured. In studies in which the cortisol secretion rate was concomitantly established by isotopic means the urinary 11-OHCS has been found to be more accurate than the urinary 17-hydroxycorticosteroids.[4] Several other recent publications have stressed the higher degree of accuracy of this method for the assessment of the adrenocortical activity in man.[5-7]

to a significant elevation in plasma cholesterol and lipoproteins,[11-13] and it has been suggested that the increase in plasma lipoproteins observed during mental stress results from a catecholamine-induced excessive adrenocortical secretion.[11] This view is supported by observations that in dogs the epinephrine-induced increase in plasma cholesterol and other lipid fractions is abolished by adrenalectomy and that administration of cortisol in such animals restores the plasma lipid response to epinephrine.[11-13] These studies emphasize the important role of the adrenal cortex in the development of blood lipid abnormalities in the experimental animal.

In addition to the stimulating effect on the adrenocortical activity, caffeine also stimulates catecholamine release[3,8-10] and increases the plasma FFA level.[1,2] Recently it has been shown that elevation of plasma FFA results in an increased synthesis and output of triglycerides by the liver leading to increased plasma triglyceride levels.[14]

The ingestion of caffeinated beverages can therefore lead to changes in blood lipids by several mechanisms. However, little work has been done so far on the long-term effects of caffeine ingestion on plasma lipids in the human subjects. Epidemiological studies have shown a positive correlation between coffee intake and levels of blood cholesterol and lipoproteins in coronary patients.[15] However, the direct role of caffeine in the production of lipid abnormalities in the human subject must at present await the result of further studies.

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Experimental Embryology

The Systematic and Specific Teratogenous Effects of Caffeine on Rodents

by M. Bertrand, E. Schwam, A. Frandon, A. Vagne and J. Alary

There is an imperative need for research into the possible risk of teratogenous effects from new drugs; studies should be conducted on the frequency and causes of the conditions we term spontaneous anomalies and on the significance of isolated chemoteratogenous incidents (Bertrand and Florio (1)). Studies subject to similar experimental conditions should also be conducted on the effects of substances classically used in therapy.

We chose to study the effects of caffeine because of its large general consumption -- dietetic as well as therapeutic.

In mice, foetal malformations caused by caffeine have been observed by Nishimura and Naka (2), as well as by Knoche and König (3); the latter authors observed 7 different anomalies (each mutually distinct from the others) in 745 foetal products of mothers treated orally with a dose of 50 mg/kg, and no anomalies in 561 control foetuses.

The methodological procedures used are reported elsewhere (Bertrand and Florio (1)).

A. Studies with Mice.-- Caffeine is administered by gastric sondage (catheter) from the 6th to the 18th day of gestation, in a dosage of 50 mg/kg (dosages of 100mg/kg or more are generally toxic).

The first series of tests were conducted on members of the breed 'Balb-C, CNRS' (known for their sensitivity to sexual hormones). From 8 females treated, 38 foetal products were obtained; of these, one foetus showed aplasia of the phalanges -- complete for 2 limbs and incomplete for the other two. We never observed this anomaly in the control animals (more than 1,000 foetal products examined); nor was it evident in animals treated with other substances.

We attempted in vain to reproduce this anomaly in a second series of tests, identical in method to the first series except that they were conducted on Swiss mice (the result was 116 normal foetuses from 21 females bred). The treatment did not seem to have reduced fecundity in the treated group as compared to fecundity in the control group.

B. Studies with Rats.-- We conducted comparable tests with rats of the Wistar breed.

The study of the acute toxicity that caffeine produced in female adults of this breed (of about 200 g in weight) revealed an acute toxicity level of DL_{50} in the 24th hour following oral ingestion of 320 ± 32 mg/kg.

Rats of the different groups tested received respectively 75, 100, 125, and 150 mg of caffeine per kilo. Administration was by gastric catheter. After a pretreatment of 8 days before the rat was bred, the administration of caffeine was continued throughout gestation. A control group was subjected to intubation by water only for the same periods. The first test group (75 mg/kg) received in addition 125 mg of sodium salicylate per kilo. The results are shown in the following table.

Females and foetuses studied	Control group	Caffeine (mg/kg) 75	100	125	150
Females bred (1)---					
Gravid females-----					
Embryos (2)--------					
Foetuses: total (3)----					
by gravid female----					
by female bred------					
Resorption %: late-----					
global---					
Malformed products %---					

(1) F = females
(2) Embryos found at the time of laparotomy
(3) Found at the time of sacrifice

The number of embryos corresponds to the number of verified implantations observed by eye at the time of an exploratory laparotomy conducted on the 9th or 10th day of gestation. All females were sacrificed on the 20th day of pregnancy.in such a way as to assure recovery of all foetal products; in the event of natural delivery, there frequently exists an early cannabalism -- especially directed toward the new-born which are weak or malformed.

The term 'global resorption' we apply to the difference between the average number of products obtained from the control group and from the treated animals (this is average number of products by female bred, not by female discovered to be gravid at the time of the laparotomy, for early resorptions may occur which are total in character). The term 'late resorption' we apply to the difference

between the number of embryos found at the time of the exploratory laparotomy and the number of foetal products carried to term. This distinction permits us to localize points at which resorption occurs. The knowledge of the number of products in a given gestation and of the productive history of every female bred permits us to learn whether the resorption involved all the offspring of a given female or only some of the products of a given gestation; in other words, if the degree of resorption seems linked to a maternal or to a foetal idiosyncracy.

Caffeine treatment caused an embryo-foetal resorption proportional to the dosage used. This resorption is influenced by a kind of elective sensitivity exhibited by products at the different stages of pregnancy, involving above all products of the foetal stage, as revealed by a comparison between the values for global resorption and those for late resorption.

Anomalies were analyzed qualitatively and quantitatively.

The only anomaly discovered in 512 control foetuses consisted of one small frontal protuberance corresponding to a micro-exencephalia; this anomaly, which is not exceptional among mice, is much more exceptional among rats. In the treated animals, in contrast, a study of 731 foetal products showed that: (1) the number of anomalies is proportional to the dosage; it seems to peak at about 20%; (2) the nature of these anomalies is very specific and always consists (in the 100 foetal products affected) of a more or less pronounced agenesis of the phalanges, ranging from the simple absence of a claw on a single phalanx to the absence of several phalanges (ectrodactylia); in addition, the stumps are sometimes characterized by sub-cutaneous hemorrhages. This anomaly is especially frequent on the left posterior limb.

Illustration
Ectrodactylia in mouse.
Ectrodactylia in rat.

These anomalies seem all the more specific since they occur in very significant numbers, proportional to the dosage of caffeine, and since similar anomalies were never found — neither in the 512 control foetuses examined in this study nor in the more than 2,000 control foetuses studied by us in our overall effort; nor are they found among the several thousands of foetuses examined in studies of treatment by various other substances.

One may ask what is the connection between the dosage of caffeine used here and a possible danger level for caffeine consumed under natural conditions. Using the method of Delga, we evaluated the dosage of caffeine in 10 samples of coffee used by various housewives from coffee beans of different origins. The mean value

is 1.13 g/liter (sigma = o.35). In countries where consumption is especially conspicuous, one liter of household coffee consumed per day can then produce caffeine in amounts of 20 to 25 mg/kg.

For therapeutic usage, the Codex fixes the maximal dosage for a 24-hour period at 1.50 g, a safety limit in harmony with our findings.

Resume.-- In the gestating rat, caffeine induces foetal resorptions and specific malformations (ectrodactylias) in numbers proportional to the dosage administered (75 to 150 mg/kg). The anomaly was only found once in treated mice.

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Embryologie expérimentale.

Sur un effet tératogène systématique et spécifique de la caféine chez les Rongeurs.

par M. BERTRAND, E. SCHWAM, A. FRANDON, A. VAGNE et J. ALARY.

La recherche, désormais impérative, du risque d'effet tératogène des médicaments nouveaux conduit à s'interroger sur la fréquence et le déterminisme des anomalies dites spontanées, sur la signification des accidents de chimiotératogenèse isolés [Bertrand et Florio (1*)] ainsi que sur les effets des substances thérapeutiques classiques, soumises aux mêmes conditions expérimentales.

Nous avons retenu la caféine en raison de sa large utilisation, diététique plus encore que thérapeutique.

Des malformations fœtales provoquées par la caféine ont été observées chez la Souris par Nishimura et Nakai (2*) ainsi que par Knoche et König (3*) : ces derniers auteurs observent 7 anomalies diverses (ne touchant pas les membres) sur 745 fœtus, de mères traités *per os* à la dose de 50 mg/kg, et 0 sur 561 fœtus témoins.

Les détails du protocole adopté sont donnés par ailleurs [Bertrand et Florio (1*)].

A. RECHERCHES CHEZ LA SOURIS. — La caféine est administrée par sondage gastrique, du 6e au 18e jour de la gestation, à la dose de 50 mg/kg (les doses égales ou supérieures à 100 mg/kg s'avèrent régulièrement toxiques).

Quelques essais ont été réalisés sur des sujets de la souche Balb-C du CNRS (connue pour sa sensibilité aux hormones sexuelles). Pour 8 femelles traitées, on obtient 38 fœtus dont l'un présente une agénésie des doigts complète sur 2 membres et incomplète sur les 2 autres. Nous n'avons jamais observé cette anomalie sur des animaux témoins (plus de 1000 fœtus examinés) non plus que sur des animaux traités par des substances diverses.

Nous avons tenté en vain de reproduire cette anomalie au cours d'un deuxième essai identique mais portant sur des souris Swiss (116 fœtus normaux pour 21 femelles saillies). Le traitement ne semble pas avoir diminué la fécondité par rapport aux témoins.

B. RECHERCHES CHEZ LE RAT. — Nous avons réalisé des expériences comparables sur le Rat, de souche Wistar.

La recherche de la toxicité aiguë de la caféine sur les femelles adultes (200 g environ) de cette race a donné une DL_{50} aiguë *per os* (à la 24e heure) de 320 ± 32 mg/kg.

(1*) M. Bertrand et R. Florio, *Actual. Pharmaceut.*, 1964, t. *10*, p. 521.
(2*) H. Nishimura et K. Nakai, *Proc. Soc. Exp. Biol. Med.*, 1960, t. *104*, p. 140.
(3*) Ch. Knoche et J. König, *Arzneim. Forsh.*, 1964, t. *14*, p. 415.

Les rats des divers lots traités ont reçu respectivement 75, 100, 125 et 150 mg de caféine par kilo, par sondage gastrique. Après un prétraitement de 8 jours, avant la mise à l'accouplement, l'administration est poursuivie tout au long de la gestation. Les témoins subissent l'intubation aux mêmes périodes, mais avec de l'eau. Le 1er lot (75 mg/kg) recevait en outre 125 mg de salicylate de soude par kilo. Les résultats sont exposés dans le tableau.

Femelles et fœtus étudiés	Témoins	Caféine (mg/kg)			
		75	100	125	150
F. saillies (1)	64	22	29	48	81
F. pleines	54	18	25	34	64
Embryons (2)	602	181	283	348	687
Fœtus : total (3)	512	140	188	145	258
par portée	9,4	7,7	7,5	4,2	4
par F. saillies	7,7	6,3	6,4	3	3,1
Résorption % : tardive	15	23	33,5	58	62
globale	–	18	17	61	60
Malformés %	0,1	3,5	7,9	20	19,8

(1) F = femelles.
(2) Embryons notés lors de la laparotomie.
(3) Au sacrifice.

Tableau récapitulatif.

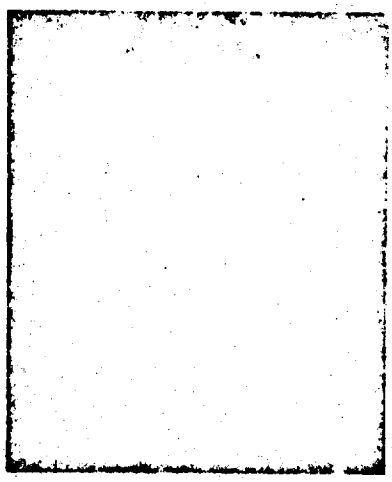
Le nombre d'embryons correspond aux implantations constatées *de visu* lors de la laparotomie exploratrice réalisée au 9e ou au 10e jour de gestation. Toutes les femelles sont sacrifiées au 20e jour de la gravidité de façon à assurer le contrôle de tous les fœtus : en effet lors d'accouchement naturel il existe souvent un cannibalisme précoce portant de préférence sur les nouveau-nés malades ou malformés.

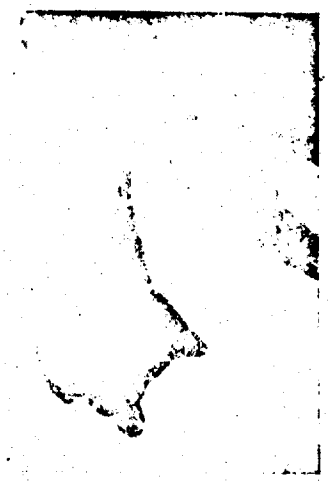
Nous appelons résorption globale la différence entre le nombre moyen de produits par femelle saillie (et non par portée, car il existe des résorptions totales précoces) chez les témoins et les traités. La résorption dite tardive est la différence entre le nombre d'embryons relevé lors de la laparotomie exploratrice et le nombre de fœtus arrivant au terme : cette distinction permet de reconnaître le stade où surviennent les résorptions. La connaissance du nombre de produits par portée et par femelle saillie permet de savoir si la résorption touche toute la portée de quelques femelles ou quelques produits par gestation, autrement dit si la résorption partielle semble liée à une idiosyncrasie maternelle ou fœtale.

Le traitement à la caféine provoque une résorption embryo-fœtale proportionnelle à la dose. Cette résorption est liée à la sensibilité élective des produits puisqu'elle atteint certains éléments de chaque portée ; elle touche surtout le stade fœtal comme le montre la comparaison entre les taux de résorption globale et tardive.

Plus spectaculaire est la constatation des anomalies tant sur le plan quantitatif que qualitatif.

L'unique anomalie relevée sur les 512 fœtus témoins consiste en une petite bosse frontale correspondant à une micro-exencéphalie : cette anomalie, qui n'est pas exceptionnelle chez la Souris, l'est beaucoup plus chez le Rat. Par contre, dans les lots traités, les examens portant sur 731 fœtus montrent que : 1) le taux d'anomalies est proportionnel à la dose, semblant plafonner vers 20 % ; 2) la nature de ces anomalies est étroitement spécifique et consiste toujours (sur les 100 fœtus atteints) en une agénésie plus ou moins prononcée des doigts allant de la simple absence d'une griffe sur un seul doigt à la disparition de plusieurs phalanges (ectrodactylie) ; les moignons sont en outre parfois porteurs d'hémorragies sous-cutanées. L'anomalie est spécialement fréquente au membre postérieur gauche.





Ectrodactylie chez la Souris.
Ectrodactylie chez le Rat.

Ces anomalies semblent d'autant plus spécifiques qu'elles surviennent à des taux très significatifs, proportionnellement à la dose de caféine, et que nous n'en avons jamais constaté de semblables non seulement sur les 512 fœtus témoins de cet essai mais encore sur plus de 2000 fœtus témoins et plusieurs milliers de fœtus traités par diverses substances lors d'autres recherches.

On peut se demander quel est le rapport entre la dose de caféine utilisée ici et la quantité risquant d'être consommée dans les conditions naturelles. Nous avons dosé la caféine (par la méthode de Delga) dans 10 échantillons de café préparés par des ménagères différentes avec des grains d'origine variée : le M.T. est de 1,13 g/l (σ = 0,35). Dans les pays fortement consommateurs, la dose ingérée lors de la consommation d'un litre de café de ménage par jour peut alors être de l'ordre de 20 à 25 mg/kg.

Sur le plan thérapeutique, le Codex fixe une dose maximale pour 24 h de 1,50 g, qui attire les mêmes constatations.

Résumé. — La caféine provoque chez la ratte gestante des résorptions fœtales et une malformation spécifique (ectrodactylie) à des taux proportionnels à la dose administrée (75 à 150 mg/kg). Cette anomalie a été retrouvée une fois chez la souris traitée.

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Teratology

Bertrand-'70
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CAFFEINE-INDUCED ECTRODACTYLY IN RODENTS
Role of Specific and Genetic Factors

By M. BERTRAND, J. GIROD and M.F. RIGAUD

In a previous publication [Bertrand et al.(1)], we showed that caffeine causes, in the gestating rat, fetal resorptions and a specific malformation, ectrodactyly, at rates proportional to the dose administered: the rate of anomalies reaches 20% with a dose of 125 mg/kg. A single case was observed in the treated mouse.

Since then, Georges and Denef (2) detected digital malformations in the rat fetus following treatment with other xanthic derivatives, especially aminophyllin.

Caffeine, moreover, has mutagenic effects on various organisms and on human cell cultures [Ostertag et al., (3)].

We thought it interesting to investigate whether these anomalies were found regularly on various lines of rats, in other rodents and, finally, whether a mutagenic effect was involved.

A. RESEARCH ON VARIOUS LINES OF RATS. - All these tests were performed on Wistar rats, from various sources, with a dose of 125 mg/kg (since this had been the dosage producing the most marked effects during previous testing), administered from the 2nd to the 15th day of gestation. However, two tests (Nos. 5 and 6) included in addition a batch treated at 25 mg/kg. The fetuses were removed, in utero, on the 20th day.

The results are shown in chronological order in the following table:

Test	Impregnated Females	Empty Females*	Normal Offspring	Ectrodactylies	Source
1	48	14	116	29	B
2	27	14	71	0	A
3	31	11	140	28	B
4	15	3	102	2	C
5**	16-7	2-0	126-71	10-0	D
6**	17-10	0-0	168-119	20-0	C

*Upon sacrifice
**Additional test at 25 mg/kg.

(1) M. Bertrand, E. Schwam, A. Frandon, A. Vagne & J. Alary, C.R. Soc. Biol., 1965, v. 159, p. 2199
(2) A. Georges & J. Denef, Arch. int. Pharmacodyn., 1968, v. 172, p. 219.
(3) W. Ostertag, E. Duisberg & M. Sturmann, Mutation Res., 1965, v. 2, p. 293.

It can be noted that ectrodactyly is found regularly with the three successive tests carried out on subjects from breed B, as well as on the rats from breed C (National Center for Scientific Research), the source of sires B.

Ectrodactyly, then, would appear more exceptional in the two other lineages of Wistar rats.

The genotype would therefore seem to condition sensitivity to the action of caffeine in the female rat.

B. RESEARCH ON THE MOUSE. - Two tests performed on 39 subjects of the same line as those of the 1st (Balb C), at doses of 50 and 75 mg/kg of caffeine administered from the 5th to the 18th day, showed a high rate of embryonic resorption (70 to 75%), but no ectrodactyly on the 23 full-term fetuses.

A 4th test carried out on 21 Swiss mice at 50 mg/kg, revealed neither embryotoxicity nor ectrodactyly in the 116 fetuses obtained.

This problem then became the object of a group experiment* carried out on 150 Swiss female mice at 75 mg/kg: From the 439 fetuses obtained neither embryonic resorption nor a single case of ectrodactyly was observed.

C. RESEARCH ON THE FEMALE RABBIT. - Fourteen female rabbits of common strain and various origins received daily doses of 100 mg/kg of caffeine through the digestive tract from the 1st to the 25th day of gestation. From the 64 products obtained, 6, from 3 litters, showed ectrodactyly continuously represented by the absence of a thumb on the front feet (confirmed by coloration of the skeleton with alizarine).

D. RESEARCH ON A POSSIBLE MUTAGENIC EFFECT IN THE RAT. - Six female and 3 male ectrodactylic rats (from rat test #6) were coupled. Of the 60 offspring obtained (certain females having had two successive gestations), none showed ectrodactyly.

SUMMARY. - The sensitivity of the female rat to teratogenous effects of caffeine seems linked to a genetic predisposition. It would appear to be a question of somations rather than mutations.

Digital anomalies induced by caffeine were found in the female rabbit but not in the mouse.

(Laboratory of Reproduction Pathology, Lyon Veterinarian School).

*Experimental teratogenesis: Study of caffeine in the mouse, Thérapie, 1969, v. 24, p. 575.

Bertrand-70
Bib. No. 268

C. R. Soc. Biol. 164(7):1488-1489. 1970.

Tératologie.

Ectrodactylie provoquée par la caféine chez les Rongeurs. Rôle des facteurs spécifiques et génétiques.

par M. Bertrand, J. Girod et M. F. Rigaud.

Dans une précédente publication [Bertrand et coll. (1)], nous avons montré que la caféine provoque, chez la ratte gestante, des résorptions fœtales et une malformation spécifique, l'ectrodactylie, à des taux proportionnels à la dose administrée : le taux d'anomalies atteint 20 % à la dose de 125 mg/kg. Un cas unique a été observé chez la souris traitée.

Depuis, Georges et Denef (2) ont retrouvé des malformations digitales chez le fœtus du Rat après traitement par d'autres dérivés xanthiques, et, particulièrement, l'aminophylline.

Par ailleurs, la caféine présente des effets mutagènes sur divers organismes et sur les cultures cellulaires humaines [Ostertag et coll. (3)].

Il nous a semblé intéressant de rechercher si ces anomalies se retrouvaient régulièrement sur diverses lignées de rats, chez d'autres Rongeurs et, enfin, s'il s'agissait d'un effet mutagène.

A. Recherches sur diverses lignées de rats. — Tous ces essais ont été réalisés sur des rats Wistar, de provenances diverses, avec la dose de 125 mg/kg, ayant présenté les effets les plus marqués au cours des essais précédents, administrée du 2e au 15e jour de la gestation. Cependant, deux essais (Nos 5 et 6) ont comporté, en outre, un lot traité à la dose de 25 mg/kg. Les fœtus sont prélevés, *in utero*, le 20e jour.

Les résultats sont exposés par ordre chronologique, dans le tableau suivant :

Essai	Femelles saillies	Femelles vides (*)	Produits normaux	Ectrodactyles	Origine
1	48	14	116	29	B
2	27	14	71	0	A
3	31	11	140	28	B
4	15	3	102	2	C
5	16	2	126	10	D
(**)	7	0	71	0	
6	17	0	168	20	B
(**)	10	0	119	0	

(*) Au sacrifice.
(**) Essai complémentaire à la dose de 25 mg/kg.

(1) M. Bertrand, E. Schwam, A. Frandon, A. Vagne et J. Alary, *C. R. Soc. Biol.*, 1965, t. *159*, p. 2199.
(2) A. Georges et J. Denef, *Arch. int. Pharmacodyn.*, 1968, t. *172*, p. 219.
(3) W. Ostertag, E. Duisberg et M. Sturmann, *Mutation Res.*, 1965, t. *2*, p. 293.

On constate que l'ectrodactylie se retrouve régulièrement lors des trois essais successifs réalisés avec les sujets de l'élevage B, ainsi que sur les rattes de l'élevage D (Centre National de la Recherche Scientifique), dont sont issus les géniteurs B.

Elle semble plus exceptionnelle dans les deux autres lignées de rats Wistar.

Il semble donc que le génotype conditionne la sensibilité à l'action de la caféine chez la ratte.

B. Recherches chez la souris. — Deux essais réalisés sur 39 sujets de la même lignée que ceux du 1er (Balb C), aux doses de 50 et 75 mg/kg de caféine administrées du 5e au 18e jour, ont montré un taux élevé de résorption embryonnaire (70 à 75 %), mais pas d'ectrodactylie sur les 23 fœtus venus à terme.

Un 4e essai réalisé sur 21 souris Swiss, à la dose de 50 mg/kg, n'a révélé ni embryotoxicité, ni aucun cas d'ectrodactylie, sur les 116 fœtus obtenus.

Ce problème a fait alors l'objet d'un travail de groupe (*), réalisé sur 150 femelles Swiss, à la dose de 75 mg/kg : il n'a été observé ni résorption embryonnaire, ni aucun cas d'ectrodactylie, sur les 439 produits obtenus.

C. Recherches chez la lapine. — 14 lapines de race commune, d'origines diverses, ont reçu quotidiennement, du 1er au 25e jour de la gestation, 100 mg/kg de caféine, par voie digestive. Sur les 64 produits obtenus, 6, issus de 3 portées, présentaient une ectrodactylie constamment représentée par l'absence de pouce aux membres antérieurs (confirmée par la coloration du squelette à l'alizarine).

D. Recherches d'un éventuel effet mutagène chez le rat. — 6 femelles et 3 mâles ectrodactyles (issus de l'essai rat n° 6) ont été accouplés entre eux. Sur les 60 produits obtenus (certaines femelles ayant eu deux gestations successives), aucun ne présentait d'ectrodactylie.

Résumé. — La sensibilité de la ratte aux effets tératogènes de la caféine paraît liée à une prédisposition génétique. Il semble s'agir de somations et non de mutations.

Des anomalies digitales induites par la caféine ont été retrouvées chez la lapine, mais non chez la souris.

(*Laboratoire de Pathologie de la Reproduction, Ecole vétérinaire, Lyon*).

(*) Tératogenèse expérimentale : Etude de la caféine chez la Souris, *Thérapie*, 1969, t. 24, p. 575.

Borker-"65
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COFFEE AND TEA

Spectral-Chromatographic Procedure for Caffeine

By EMANUEL BORKER and KATHERINE G. SLOMAN (General Foods Technical Center, White Plains, N.Y.)

A spectral-chromatographic procedure for the determination of caffeine, based on a modification of the Levine procedure, was submitted for collaborative study. Results from eight collaborators indicate that the method is accurate for coffee products and a beverage sirup. For decaffeinated soluble coffee the method is more accurate and more precise than the Bailey-Andrew methods. However, it is not as precise as the Bailey-Andrew methods for regular soluble coffee or for roasted and ground coffee.

Since a bias due to noncaffeine nitrogen has been demonstrated in the official Bailey-Andrew procedure for caffeine in decaffeinated coffee products (1), a more accurate method is needed. A new official method should be applicable to all types of coffee samples and, if possible, to other beverages containing caffeine. Two spectral-chromatographic methods that were briefly studied in previous work, the Yeransian procedure (1) and a modified Levine procedure (2), showed promise as accurate general methods for caffeine in coffee products (3).

A more extensive comparative study of the two spectral methods and the micro Bailey-Andrew method (4) was completed in the Associate Referee's laboratory. Results are shown in Table 1. Each result represents the average of eight determinations for the chromatographic procedures and six determinations for the Bailey-Andrew method. The chromatographic procedures were performed by two analysts, each in duplicate on two different days. The Bailey-Andrew analyses were performed in duplicate by three different analysts. The standard deviations were calculated from these few determinations to provide an estimate of the comparative precision of the three methods.

There is good agreement between the Bailey-Andrew and modified Levine procedures for regular coffee samples. The Yeransian method consistently gives values slightly higher than the other two methods and has the poorest precision of the three methods.

Table 1. Recovery of caffeine by three methods (per cent dry basis)

Sample	% Caffeine		
	Bailey-Andrew Method	Modified Levine Method	Yeransian Method
Regular Coffee			
Green 1	1.58±.02	1.57±.06	1.65±.12
Green 2	1.57±.03	1.61±.04	1.68±.07
Roasted	1.47±.03	1.47±.05	1.61±.06
Soluble	3.31±.09	3.37±.08	3.43±.12
Decaffeinated Coffee			
Green	0.041±.003	0.047±.001	0.049±.002
Roasted	0.060±.009	0.055±.001	0.051±.004
Soluble	0.154±.015	0.088±.001	0.081±.004

For the decaffeinated coffees, the Yeransian procedure yields values that are closer to the true values than those by the Bailey-Andrew method (2). The modified Levine method gives values similar to those obtained by the Yeransian procedure, but with better precision.

Since the Levine procedure is the simplest of the three procedures investigated and since it accurately measures the caffeine content of all types of coffees, this method should be suitable for routine analysis of coffee. Its excellent possibilities prompted an investigation of the possible sources of error in the modified Levine method. A basic extraction medium, rather than the acid originally suggested by Levine, was chosen because the Levine method gave somewhat higher values than the Bailey-Andrew

method in the first trial. Differential spectrophotometry of the final extracts obtained by both the Levine methods showed the presence of a greater amount of impurity in the extracts from the acid extraction than in the extracts from the basic extraction. Caffeine alone was carried through both Levine procedures; by the basic extraction procedure 99% of the starting caffeine was recovered and by the acid procedure 97% was recovered. All comparative results were, therefore, obtained by the modified or basic Levine procedure as described below.

Variation in either volume or flow rates, as well as amounts, strength of reagent added, and time of storage of the absorbents, were all investigated for possible sources of error in the Levine procedure. The most critical point is the amount of acid used; if this is below the amount required by the procedure the amount of caffeine found will be low. Too much acid, too little, or too much base in a ± 50% range do not appear to affect the results significantly. The amount of ether used should not vary more than ± 25 ml. Several solvents, among them butyl ether, carbon tetrachloride, and trichlorethylene, were investigated as possible substitutes for the flammable ether; none appeared suitable for this purpose. The regular coffee samples must be diluted with ammonia (1 + 2) after initial extraction for accurate results. If they are diluted with water, the column extraction is not effective.

An attempt to apply the chromatographic method to tea and tea products gave results that were sometimes grossly different from those obtained by the Bailey-Andrew procedure. In general, the Levine procedure gave results which were higher than those by the Bailey-Andrew on soluble tea and erratically lower on regular tea.

Spectral-Chromatographic Determination of Caffeine

(Applicable to decaffeinated coffee)

Preparation of Samples

Grind green and roasted coffees to about 50–70 mesh. Use good coffee grinder or large Waring Blendor, and keep sample cold with Dry Ice while grinding to avoid pastiness.

Place 1.0 g ground decaffeinated coffee or 0.5 g decaffeinated sol. coffee in 100 ml beaker. Add 5 ml NH_4OH (1 + 2) and heat 2 min. on steam bath. Add 6 g Celite 545 and mix thoroly.

Preparation of Column

(a) *Acid column.*—Place fine glass wool plug in base of 25 × 250 mm column. Add 2 ml 4*N* H_2SO_4 to 2.0 g Celite 545 and mix well by kneading with spatula blade. Transfer to column, using powder funnel, and tamp, using gentle pressure, to uniform mass. Place small glass wool wad above surface. Insert tip into 50 ml vol. flask.

(b) *Basic column.—Layer A.*—Mix 3 g Celite 545 and 2 ml 2*N* NaOH, and place in 25 × 250 mm column over glass wool plug as above. *Layer B.*—Transfer sample plus Celite mixt. to column directly over layer A. Dry-wash beaker with ca 1 g dry Celite, transfer to column, and tamp to uniform mass.

(Both acid Celite and basic Celite mixts may be prepd in large batches and used as needed. In this case, use 4.2 g acid mixt. and 5 g base mixt./column.)

Determination

Mount basic column above acid column. Pass 150 ml H_2O-satd ether thru basic column to acid column. Then pass 50 ml ether thru acid column. Place 50 ml vol. flask under acid column. Pass 50 ml H_2O-satd $CHCl_3$ over acid column, washing tip of basic column with first portion. Dil. contents of vol. flask to 50 ml with H_2O-satd $CHCl_3$, mix, and read absorbance at 276 mμ against $CHCl_3$ blank.

Prep. caffeine stds contg 0.25, 0.5, and 0.75 mg/50 ml in H_2O-satd $CHCl_3$. Establish absorptivity of stds for spectrophotometer used, and check occasionally. Calc. caffeine in samples from absorptivity of stds.

Collaborative Study

The Levine procedure in its modified form was submitted to a collaborative study. The samples chosen for this study were decaffeinated soluble coffee, roasted and ground coffee, regular soluble coffee, and a beverage sirup. Since the chromatographic procedure was new to the collaborators for coffee analysis, it was suggested they familiarize themselves with the technique by using the soluble coffee sample. The expected range for this sample was supplied as a reference point.

Most collaborators reported no difficulty with the procedure. One reported that a 20% increase in both acid and Celite was needed in the acid column to approach the value of the trial sample. Another collaborator reported poor caffeine recovery from solutions of caffeine treated as for regular coffee and therefore did not analyze the samples submitted. It was suggested that a stock solution containing 100 mg caffeine per 100 ml chloroform be used to make the standard caffeine dilutions.

Results received from the collaborators are summarized in Table 2. The average of

Table 2. Collaborative results for caffeine recovery from various coffee samples by the modified Levine procedure

Coll.	% Caffeine			
	Decaffeinated Soluble	Roasted and Ground	Regular Soluble	Beverage Sirup
A	0.074	1.31	2.63	0.055
	0.083	1.18	2.83	0.059
B	0.089	1.70	3.46	0.060
	0.093	1.68	3.42	0.067
C-1	0.078	1.46	3.37	0.066
	0.078	1.45	3.35	0.066
C-2	0.080	1.46	3.35	0.065
	0.080	1.47	3.38	0.065
D	0.039[c]	1.39	2.93	0.060
	0.039[c]	1.23	2.95	0.062
	0.062[c]	1.25	2.89	0.060
E	0.072	1.32	3.12	0.064
	0.078	1.46	3.14	0.066
F-1	0.082	1.47	3.30	0.069
	0.084	1.48	3.39	0.069
F-2	0.081	1.56	3.56	0.070
	0.072	1.47	3.07	0.062
F-3	0.081		3.39	0.066
G[a]	0.074	1.43	3.04	0.069
	0.074	1.51	3.15	0.064
H	0.084	1.45	3.04	0.069
	0.083	1.32	2.98	0.070
Av. No. Detns	19	21	22	22
Av. ± Std Dev.	0.080 ± 0.0055	1.43 ± 0.13	3.17 ± 0.24	0.065 ± 0.0040
Coeff. of var., %	±6.9	±9.1	±7.6	±6.2
Micro Bailey-Andrew value[b]	0.14	1.40	3.23	0.066[d]

[a] Increased level of acid and Celite used in acid column.
[b] Associate Referee's Laboratory.
[c] Value not included in average since wrong dilution was used; absorbance value too low to be corrected reliably.
[d] Added level.

all values obtained, except that for decaffeinated soluble coffee, agrees well with the value obtained by the official method. The caffeine content of decaffeinated soluble is lower by the Levine procedure because of the non-caffeine interference in the Bailey-Andrew procedure.

The coefficient of variation found for the macro and micro Bailey-Andrew procedures in previous collaborative studies (5, 6) was compared with the coefficient of variation found for the Levine method in this study (Table 3). The Levine procedure is more accurate and more precise than the micro Bailey-Andrew when it is applied to the decaffeinated soluble coffee. This variation in the Bailey-Andrew procedure is probably caused by erratic extraction of nitrogenous material, not caffeine. The Levine method is not as precise as the Bailey-Andrew on regular ground or soluble coffee.

The Power-Chesnut method is an official method for coffee and tea (7). However, it is not applicable to coffee extracts or soluble coffee. These products now represent a substantial factor in the trade. The Associate Referee knows of no laboratories now using this method. Few, if any, references are made to this procedure in the current literature, and no data obtained by the method have been reported recently.

Table 3. Collaborative data on caffeine recovery by three methods (comparison of precision)

Year of Study	Bailey-Andrew (Macro) Method		Bailey-Andrew (Micro) Method		Levine (Mod.) Method	
	Av.	% Coeff. of Var.	Av.	% Coeff. of Var.	Av.	% Coeff. of Var.
Roasted and Ground Coffee						
1959	1.32	5.3	1.32	2.3		
1964					1.43	9.1
Regular Soluble Coffee						
1959	3.48	5.5	3.61	3.0		
1960	3.06	3.9	3.10	2.6		
1964					3.17	7.6
Decaffeinated Soluble Coffee						
1959	0.121	9.9	0.132	8.3		
1960	0.11	13.6	0.12	12.5		
1964					0.080	6.9

Recommendation

It is recommended—

(1) That the spectral-chromatographic method as described be adopted as official, first action for decaffeinated coffee products.

(2) That the applicability of the spectral-chromatographic method to other coffee be noted, with reference to the greater variability than that found in the Bailey-Andrew procedures.

(3) That all references to analysis of decaffeinated coffee or products low in caffeine in methods **14.019**, **14.020**, **14.021**, and **14.044** be deleted.

(4) That the Power-Chesnut method, **14.019** and **14.043**, be declared surplus.

(5) That the spectral-chromatographic method be referred to the General Referee on Flavors and Nonalcoholic Beverages for consideration as a replacement or alternative for the present method, **8.032–8.036**.

(6) That further study be made on application of the spectral-chromatographic method to tea products.

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The recommendations of the Associate Referee, except recommendation (2), were approved by the General Referee and by Subcommittee C, and were accepted by the Association. See *This Journal*, **48**, 171–172 (1965).

D. M. Smith, Department of National Health and Welfare, Ottawa, Ontario

I. A. Striffler, The Nestle Co., Marysville, Ohio

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(7) *Official Methods of Analysis*, 9th Ed., sec. **14.019**.

This report of the Associate Referee, Emanuel Borker, was presented at the Seventy-eighth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 19-22, 1964, at Washington, D.C.

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The Acute Oral Toxicity of Caffeine

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The signs of caffeine poisoning in man are summarized by Gemmill (1958) as insomnia, nervousness, headache, nausea, vomiting, tachycardia, premature systoles, augmented respiration, and muscular tremors, the fatal dose being estimated at over 0.14 g/kg. Estimates of the oral minimal lethal dose, median lethal dose, or simply "lethal dose" in various laboratory animals, listed by Spector (1956), lie in the range of 0.1–0.36 g/kg. Signs of caffeine poisoning recorded in mice by Fühner (1932) are excitement, epileptiform convulsions, anorexia, stiff muscles, and death due to respiratory failure. In the rat, Maloney (1935) lists apprehensiveness, tremors, a staggering gait, distressed breathing, and a state of depression, and Holck (1949) adds loss of weight, restlessness, excitement, and confusion. In rabbits, cats, and/or dogs, Salant and Rieger (1909) record weakness, loss of weight, diarrhea, and inflammation of the stomach and intestines; Salant (1911), a fall in blood pressure; and Sollmann and Pilcher (1911), strychnine-like convulsions and cardiac arrest.

The study herein reported was concerned with an assessment of the clinical and pathological signs of poisoning in animals receiving caffeine in the range of the oral median lethal dose. Preliminary experiments on the dog revealed that a dose of 0.200 g/kg produced excitement, anorexia, and a delayed vomiting. Vomiting began some 2 hours after drug administration and continued at intervals for 24 hours, the vomitus being coffee-colored. The remaining work was performed upon the albino rat in which vomiting cannot occur.

[1] The author wishes to acknowledge the technical assistance of Jack Coates, J. Marion A. Price-Jones, Patricia E. Sheppard, and Howard D. Steele, and the receipt of a grant in aid of this project through Woodard Research.

METHOD

Female albino rats were assembled in metabolism cages, one rat per cage, food was withheld for 16 hours, caffeine was administered in aqueous solution by stomach tube, and the observations noted by Boyd (1959) were made. Caffeine BP, USP was dissolved in distilled water immediately before administration in a volume of 2.0 ml per kilogram body weight. The doses of caffeine expressed as grams per kilogram body weight, and number of animals per dose (in parentheses) were: 0.000 (20), 0.160 (8), 0.180 (16), 0.200 (8), 0.220 (8). The animals were 3-6 months of age when used and averaged (± S.D.) 203 ± 28 g in body weight.

RESULTS

The median lethal dose (± S.E.) was calculated to be 0.192 ± 0.018 g per kilogram body weight. The oral median lethal dose of caffeine in rats has been reported as "approximately" 0.200 g/kg by Smith and Hambourger (1935), and as 0.233 ± 0.014 g/kg by Scott and Chen (1944).

There were nineteen survivors and twenty-one deaths. Death time was rather uniform at an average (± S.D.) of 30.0 ± 9.6 hours after administration of drug.

Clinical signs in the group of rats which recovered were as follows. One hour after drug administration the animals showed lack of curiosity to sound or sight, on mechanical stimulation they showed hyperreflexia, but had little inclination to walk or move. In brief, they were weak, tense, and withdrawn. When moving, they were unsteady on the feet and their movements were ataxic. Some quarter of the animals adopted brief cataleptic stances and about the same number gave evidence of being in pain (phonation on mild mechanical stimulation). By 6 hours, half the rats had developed varying degrees of cataplectic resistance to movement and a red crusting about the inner canthus of the eye. By 12 hours, the eyelids were swollen and inflamed. At 24 hours, 80% of the animals were in the same clinical state, with loose stools or slight diarrhea and perineal soiling; two rats were normal in clinical appearance and two had tremors, pallor, and were moderately excited. Half the rats were normal at 48 hours, two were excited, and two had bouts of clonic convulsions lasting a few seconds each. At 72 hours all of this group of rats had a normal clinical appearance. Data upon measured clinical signs are summarized in Table 1 and indicate that the clinical syndrome described above was accompanied at 24 hours by anorexia, loss of body weight, a tendency to take drinking of less water, and some fluctuation in body temperature.

In the group of rats which died, the clinical syndrome began in a manner similar to that described above. Clinical deterioration was progressive from the tenth hour until death. The animals ate no food and drank no water. Diarrhea became profuse and loss of body weight was marked.

TABLE 1

BODY WEIGHT, FOOD INTAKE, WATER INTAKE, URINE OUTPUT, AND COLONIC TEMPERATURE OF ALBINO RATS FOLLOWING ORAL ADMINISTRATION OF CAFFEINE IN THE RANGE OF THE MEDIAN LETHAL DOSE[a]

Group	No. of animals	Days after caffeine 1	2	3
Body weight: grams increase (+) or decrease (—)				
Controls	20	+13 ± 4	+14 ± 5	+13 ± 6
Survivors	19	— 7 ± 9	— 8 ± 12	+ 1 ± 14
Nonsurvivors	21	—16 ± 5	—22 ± 5	—
Food intake: grams chow per kilogram body weight per 24 hr				
Controls	20	93 ± 23	93 ± 24	82 ± 24
Survivors	19	16 ± 20	46 ± 28	79 ± 26
Nonsurvivors	21	0 ± 0	0 ± 0	—
Water intake: milliliters per kilogram body weight per 24 hr				
Controls	20	183 ± 51	127 ± 32	124 ± 25
Survivors	19	59 ± 67	138 ± 60	183 ± 74
Nonsurvivors	21	0 ± 0	0 ± 0	—
Urine output: milliliters per kilogram body weight per 24 hr				
Controls	20	39 ± 31	38 ± 18	37 ± 15
Survivors	19	26 ± 23	65 ± 28	82 ± 43
Nonsurvivors	21	10 ± 9	1 ± 3	—
Colonic temperature: °F				
Controls	20	98.9 ± 0.8	99.1 ± 0.9	99.6 ± 1.1
Survivors	19	98.1 ± 2.8	99.9 ± 1.1	100.0 ± 1.5
Nonsurvivors	21	93.7 ± 3.2	94.9 ± 4.0	—

[a] The results are expressed as mean ± standard deviation.

They became anuric. Body temperature fell. These, plus the clinical signs described above, persisted until death. Two-thirds of the animals died of respiratory failure following tetanic convulsions. The remaining deaths were due to cardiovascular collapse.

The gross appearance of the organs at autopsy suggested the presence of extensive inflammation of the gastrointestinal tract and toxic damage to the liver, kidneys, spleen, adrenals, thymus gland, and lungs. The

cardiac portion of the stomach, which is lined by stratified squamous epithelium, was distended with gas, and, in some 20% of cases, there were small areas of inflammation at or near its junction with the glandular portion of the stomach. The pyloric or glandular part of the stomach was pale with numerous (4 to 24) small areas of inflammation and hemorrhage. The duodenum, jejunum, and ileum were obviously inflamed in some 40% of the animals which died. The duodenum and jejunum were moderately distended with a yellow-stained fluid which occasionally contained blood. The amount of succus entericus which could be expressed from the ileum was obviously reduced in 20% of the animals. There were intussusceptions of the ileum in two rats. The cecum was distended with a yellow fluid. The colon appeared normal in most animals, occasionally slightly inflamed.

There were areas of pallor over the liver. The kidneys and spleen were pale. The mesentery and omentum were occasionally yellow-stained. The adrenal glands were enlarged and dark red in color and the thymus was reddish mauve, in contrast to its normal white color. The atria of the heart were engorged with blood in a majority of rats. In all animals the lungs were of a reddish-brown color and often obviously enlarged. The ovaries, skeletal muscle of the ventral abdominal wall, submaxillary salivary glands, brain, skin, and homogenized residual carcass appeared normal.

Shifts in the wet weight and water levels of organs and tissues are summarized in Table 2. Values in the control animals were not significantly different from control values reported by Boyd (1959) and have been omitted from Table 2. All of the animals had lost weight at the time of death. Controls sacrificed at the same times had regained body weight lost during the initial period of restriction of food intake. The body weight of rats dying from caffeine poisoning averaged 14.5% less than that of the controls. Part of the loss of body weight was due to reduction in the amount of food and products of digestion of food in the gastrointestinal tract. As shown in Table 2, the relative distribution of weight had shifted in the bodies of these animals. There was a considerable loss of weight in the tissues of the gastrointestinal tract, in the liver, and in the spleen. The adrenal glands and lungs gained weight, absolutely and relatively to autopsy body weight. Changes in the absolute weight of heart, ovaries, and residual carcass (the residuum of the body after removal of the organs listed in Table 2) were so small that these

tissues comprised a relatively greater proportion of autopsy body weight than was the case in the control rats.

Most of the organs of the body were more dry than normal. Drying was particularly evident in mesentery-omentum, the submaxillary salivary

TABLE 2

MEAN SHIFTS[a] IN THE WET WEIGHT[b] AND WATER LEVELS[c] OF ORGANS AND TISSUES OF ALBINO RATS AT AUTOPSY FOLLOWING DEATH DUE TO ORAL ADMINISTRATION OF A LETHAL DOSE OF CAFFEINE

Organ or tissue	Wet weight		Water level	
Spleen	—58.0	(<0.001)	+ 3.1	(0.05)
Liver	—43.0	(<0.001)	— 6.4	(0.02)
Jejunum	—27.3	(<0.001)	+ 4.5	(0.6)
Colon	—26.3	(<0.001)	—12.5	(<0.001)
Submaxillary glands	—25.3	(<0.001)	—21.5	(0.001)
Pyloric stomach	—24.4	(<0.001)	+ 2.2	(0.4)
Ileum	—21.9	(0.02)	+ 8.3	(0.1)
Cecum	—15.8	(0.02)	— 1.7	(0.8)
Duodenum	—15.3	(0.2)	— 2.6	(0.4)
Thymus gland	—14.6	(0.2)	— 2.5	(0.6)
Kidneys	— 3.2	(0.6)	— 3.5	(0.4)
Cardiac stomach	— 1.4	(0.8)	— 3.1	(0.3)
Mesentery-omentum	— 1.2	(0.9)	—27.2	(0.01)
Skin	+ 0.5	(0.9)	—20.1	(<0.001)
Brain	+ 6.5	(0.1)	0.0	(1.0)
Abdominal wall muscle	+ 6.6	(0.1)	— 8.8	(0.025)
Residual carcass	+10.3	(<0.001)	— 9.4	(0.02)
Ovaries	+16.7	(0.025)	—15.4	(0.005)
Heart	+20.1	(0.001)	— 2.9	(0.1)
Lungs	+47.4	(0.005)	—11.8	(<0.001)
Adrenal glands	+67.9	(0.001)	+11.9	(0.005)

[a] Expressed as a mean percentage change from the controls, specifically $100\,[(\bar{X}d - \bar{X}c) / \bar{X}c]$, where $\bar{X}d$ is the mean of the drug-treated animals and $\bar{X}c$ is the mean of the controls. In parentheses is the probability (P) that $\bar{X}d - \bar{X}c$ equals zero by a t test.

[b] Calculated as a percentage of body weight at autopsy.

[c] Calculated as grams water per 100 g dry weight of tissue.

glands, the skin, the ovaries, the colon, the lungs, the liver, skeletal muscle of the ventral abdominal wall, and residual carcass. A significant increase in water level was found in the adrenal glands, possibly in the spleen.

Histopathologic observations were as follows. Capillaries and veins in the lamina propria were dilated and engorged with blood in all areas

the gastrointestinal tract from the pyloric stomach to the cecum. Accompanying the congestion there were varying degrees of necrosis of the glandular epithelium and some hemorrhage. The goblet cells were numerous and enlarged. The sinusoids of the liver were distended with packed

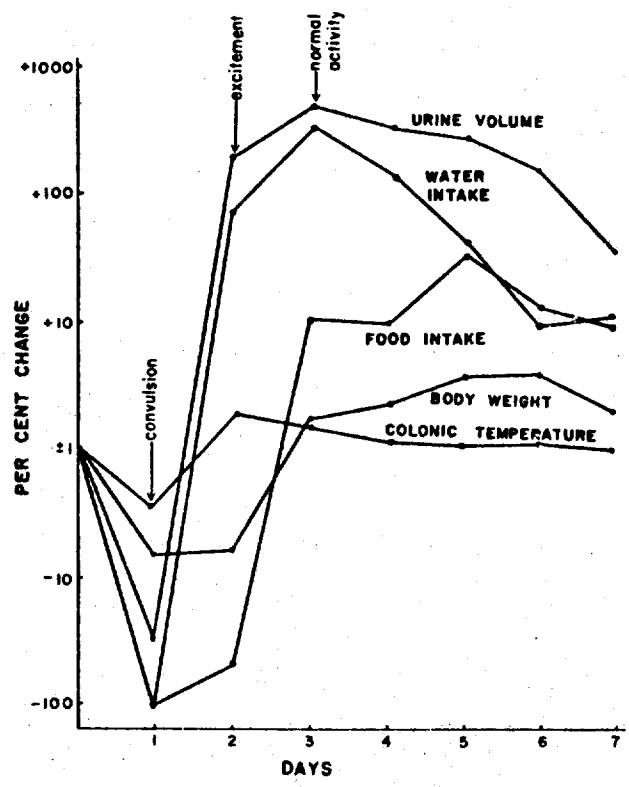

FIG. 1. Percentage changes in body weight, food intake, water intake, urine output, and colonic temperature of a rat which survived oral administration of caffeine in a dose of 0.220 g per kilogram body weight.

erythrocytes and the hepatic cells were shrunken and pale-stained. The brush border of cells lining the renal proximal convoluted tubules appeared necrotic, red blood cells were packed in capillaries in the region of the loops of Henle, and cells lining the distal convoluted tubules had swollen to seal off the lumen.

The periphery of the spleen appeared contracted, with erythrocytes

forced out of the sinuses and into the central sinuses where they were tightly packed, compressing the cords of Billroth and reticulum of the red pulp. There was a deficiency of zymogenic granules in the acinar glands of the pancreas and a deficiency of blood in the capillaries of the islets of Langerhans. The thymus gland appeared normal except for occasional areas of necrosis of the reticular cells. Sinusoids of the zona glomerulosa and zona fasciculata of the adrenal cortex were full of packed red blood cells. Capillaries in cardiac muscle of the auricles and ventricle and in the alveolar walls of the lung were dilated and filled with packed blood cells. The remaining organs appeared normal histologically.

Rats which survived the initial toxic effects of these doses of caffeine were observed for 6 weeks. There were no delayed deaths in this period. Recovery was accompanied by a marked increase in urine output and water intake, as exemplified in Fig. 1. There was some increase in appetite and gain in body weight. All measurements were returning toward normal by the end of 1 week.

SUMMARY

The oral median lethal dose of caffeine in albino rats was estimated to be (±S.E.) 0.192 ± 0.018 g per kilogram body weight. The clinical signs of poisoning from these doses were schizophreniform withdrawal, hyperreflexia, vertigo, ataxia, evidence of pain, cataleptic stances, cataplexy, diarrhea, anuria, anorexia, adipsia, hypothermia, blepharitis, and loss of body weight. Death occurred in (±S.D.) 30 ± 9.6 hours and was immediately due to respiratory failure following tetanic convulsions or to cardiovascular collapse. Autopsy revealed the presence of a fulminating gastroenteritis, congestion of the lungs, hepatitis, nephritis, toxic effects upon the heart, spleen, pancreas, thymus gland, adrenal glands, and dehydration of many organs and tissues. Survival was characterized by a marked polydipsia and polyuria.

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The Acute Oral Toxicity in Guinea Pigs of Acetylsalicylic Acid, Phenacetin, and Caffeine, Alone and Combined

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Boyd (1959a–d) has shown that the acute oral toxicity in albino rats of a mixture of acetylsalicylic acid, phenacetin, and caffeine is, in general, a summation of the corresponding toxicity of the individual ingredients of the mixture. According to Smith (1958), there is no corresponding evidence available on any other species except the guinea pig. Loewe *et al.* (1927) analyzed, by means of isoboles, data upon the threshold ("Schwelle sichtbarer Allgemeinwirkungen") and lethal ("tödliche Grenzdosis") doses of acetylsalicylic acid, phenacetin, and codeine, given orally to guinea pigs, and offered some evidence of antagonism of toxic effects. The presently described work was undertaken to determine if similar antagonism could be found in a mixture of acetylsalicylic acid, phenacetin, and caffeine given orally to guinea pigs.

METHODS

The experiments were performed upon young, male, CBL guinea pigs, weighing 300–500 g. The numbers of animals used are indicated in Table 1. While under close observation, they were placed in metabolism cages, food was withheld for 16 hours prior to oral administration of the drug, and subsequent measurements were made as described by Boyd (1959a). The results are summarized in Tables 1–3.

RESULTS

The clinical signs of acute toxicity to acetylsalicylic acid in the guinea pig were the same as reported by Boyd (1959a) in albino rats except that rhinorrhagia did not occur and there was little diarrhea. Death was due to respiratory failure preceded by generalized convulsions or prostration in most animals, occasionally to cardiovascular shock. The $LD_{50} \pm SE$

TABLE 1

A COMPARISON OF CHANGES IN BODY WEIGHT, FOOD AND WATER INTAKE, AND COLONIC TEMPERATURE IN GUINEA PIGS GIVEN ASA, PHENACETIN, CAFFEINE, AND APC[a]

Drug	N	Days after drug 1	2	3	4	5
Body weight: grams increase (+) or decrease (—)						
Water (controls)	38	+15 ± 13	+24 ± 12	+33 ± 15	+37 ± 15	+44 ± 16
Acetylsalicylic acid	36	—14 ± 14	— 3 ± 22	+ 1 ± 21	+ 8 ± 21	+11 ± 19
Phenacetin	37	— 8 ± 12	—18 ± 33	—11 ± 37	— 3 ± 38	+ 4 ± 31
Caffeine	37	—25 ± 10	—24 ± 25	—20 ± 34	—12 ± 39	— 4 ± 46
APC found	33	—15 ± 17	—11 ± 21	— 6 ± 26	+ 6 ± 19	+10 ± 21
APC estimate	—	—12 ± 13	—11 ± 23	— 5 ± 29	+ 2 ± 30	+ 7 ± 26
Food intake: grams Purina pellets per kilogram body wt. per 24 hr						
Water (controls)	38	76 ± 29	80 ± 17	87 ± 17	82 ± 18	82 ± 16
Acetylsalicylic acid	36	57 ± 11	73 ± 23	75 ± 21	73 ± 20	82 ± 8
Phenacetin	37	40 ± 20	62 ± 33	65 ± 25	68 ± 23	68 ± 21
Caffeine	37	26 ± 12	36 ± 31	48 ± 31	65 ± 8	73 ± 26
APC found	33	41 ± 14	65 ± 25	72 ± 30	91 ± 49	75 ± 16
APC estimate	—	48 ± 15	66 ± 28	69 ± 23	71 ± 21[b]	75 ± 14

[a] The results are expressed as mean ± standard deviation.

[b] Mean of APC estimate differs from mean of APC actual at $P < 0.05$ and at $N = 33$ for APC estimate.

TABLE 1 (*Continued*)

Drug	N	Days after drug 1	2	3	4	5
Water intake: milliliters per kilogram body wt. per 24 hr						
Water (controls)	38	123 ± 47	130 ± 39	132 ± 39	113 ± 42	112 ± 32
Acetylsalicylic acid	36	77 ± 19	156 ± 49	155 ± 30	134 ± 40	131 ± 23
Phenacetin	37	62 ± 53	124 ± 54	159 ± 76	151 ± 47	134 ± 44
Caffeine	37	45 ± 21	79 ± 69	121 ± 45	175 ± 82	173 ± 95
APC found	33	79 ± 41	117 ± 27	134 ± 37	122 ± 24	139 ± 71
APC estimate	—	68 ± 35	138 ± 52[b]	153 ± 52[b]	143 ± 45[b]	134 ± 36
Colonic temperature: °F						
Water (controls)	38	103.5 ± 1.6	103.2 ± 1.5	103.3 ± 0.8	103.2 ± 1.4	103.4 ± 1.0
Acetylsalicylic acid	36	102.9 ± 2.1	103.2 ± 1.1	103.2 ± 1.1	103.1 ± 1.2	103.8 ± 1.0
Phenacetin	37	98.9 ± 6.8	102.3 ± 2.9	103.1 ± 1.5	102.7 ± 2.7	103.4 ± 1.2
Caffeine	37	100.3 ± 2.8	101.2 ± 2.2	101.3 ± 3.1	102.8 ± 1.2	103.0 ± 1.1
APC found	33	102.2 ± 2.6	106.6 ± 4.1	102.9 ± 1.0	103.1 ± 0.9	103.5 ± 1.1
APC estimate	—	101.5 ± 4.3	102.8 ± 2.0	103.2 ± 1.4	103.0 ± 1.9	103.7 ± 1.1

was 1.19 ± 0.073 g per kilogram compared with 0.92 ± 0.045 in albino rats. In guinea pigs dying within 12 hours of drug administration, the dominant feature at autopsy was a severe gastroenteritis. When death was delayed beyond 2 days, the pathological findings were as summarized in Table 3. The latter were similar to those reported in albino rats.

TABLE 2

A COMPARISON OF THE INCIDENCE OF CLINICAL SIGNS IN GUINEA PIGS FOLLOWING THE ADMINISTRATION OF ASA, PHENACETIN, CAFFEINE, AND APC

Clinical sign[a]	Acetylsalicylic acid	Phenacetin	Caffeine	APC found	APC estimate	P (estimate equals found)
1 Hour after drug administration						
Withdrawal	56	38	21	31	46	0.4
Restlessness	38	30	39	45	34	0.6
Phonation	6	0	0	0	3	0.4
Diarrhea	3	0	9	10	2	0.2
Ataxia	0	97	0	3	45	< 0.001
Catalepsy	0	0	85	0	3	0.4
Hyperreflexia	0	0	79	0	3	0.4
Dyspnea	0	0	24	0	1	0.8
Tremor	0	0	21	0	1	0.8
Fasciculation	0	0	9	0	0	1.0
Colonic temperature: M. ± S.D.	101.9 ± 0.73	96.3 ± 3.2	99.9 ± 1.9	101.1 ± 1.3	99.3 ± 1.9	< 0.001
6 Hours after drug administration						
Restlessness	50	9	70	54	32	0.005
Withdrawal	33	79	0	14	53	< 0.001
Stupor	12	17	0	4	14	0.2
Dyspnea	12	0	0	4	6	0.7
Tremor	12	0	0	4	6	0.7
Ataxia	7	64	0	11	33	0.05
Shivering	0	27	0	0	12	0.05
Hyperreflexia	0	0	39	4	2	0.4
Fasciculation	0	0	12	0	0	1.0
Prolapse of rectum	0	0	6	0	0	1.0
Convulsions	0	0	3	0	0	1.0
Colonic temperature: M. ± S.D.	101.6 ± 1.0	92.9 ± 6.7	99.9 ± 3.4	101.5 ± 2.1	97.5 ± 3.7	< 0.001

[a] Numbers in columns 2–6 represent percentage of incidence of clinical signs except colonic temperatures, which are recorded in degrees Fahrenheit.

TABLE 3

A COMPARISON OF CLINICAL AND PATHOLOGICAL SIGNS IN GUINEA PIGS DYING FROM ADMINISTRATION OF ASA, PHENACETIN, CAFFEINE, AND APC

Observation	Acetylsalicylic acid	Phenacetin	Caffeine	APC
$LD_{50} \pm SE$ (g/kg)	1.19 ± 0.073	1.87 ± 0.074	0.230 ± 0.021	1.33 ± 0.040
Early (< 12 hr) deaths (% of total deaths)	81	28	37	50
Delayed (2 days) deaths (% of total deaths)	12	33	29	19
Premortem signs	Convulsions, mania	Hypothermia, stupor	Convulsions, stupor	Hypothermia, stupor, mania
Histopathology:				
1. G.I. Tract	Inflammation	Inflammation	Inflammation	Inflammation
2. Liver	Congestion	Degeneration	Edema	Degeneration
3. Kidneys	Necrosis	Minor	Minor	Edema
4. Heart	Minor	Minor	Congestion	Minor
5. Lungs	Edema	Edema	Edema	Edema
6. Spleen	Contraction	Minor	Congestion	Contraction
7. Adrenals	Congestion	Congestion	Congestion	Congestion
8. Pancreas	Minor	Congestion	Minor	Minor
9. Testes	Minor	Minor	Minor	Minor

The syndrome of intoxication to phenacetin in the guinea pig was also similar to that reported in albino rats by Boyd (1959b) except that exophthalmos, dacryorrhea, and sialorrhea did not appear in guinea pigs. Most of the animals died within 48 hours of respiratory failure preceded by colonic temperatures of 77–80° F. The $LD_{50} \pm SE$ was 1.87 ± 0.074 g per kilogram compared with 1.65 ± 0.35 in albino rats. Histopathology in guinea pigs dying after 2 days was similar to that reported in albino rats except that renal tubular necrosis was of a minor degree.

The $LD_{50} \pm SE$ for caffeine was found to be 0.230 ± 0.021 g per kilogram in guinea pigs. Boyd (1959c) reported 0.192 ± 0.018 in albino rats. The clinical and pathological syndrome was similar in the two species. It is of interest that intussusception of the ileum was found in some 10% of albino rats; intussusception and/or prolapse of the rectum was present in 12% of the guinea pigs. The common cause of death was respiratory failure following tonic-clonic convulsions.

The APC mixture consisted of 50 g of acetylsalicylic acid, 46 g of phenacetin, and 4 g of caffeine in each 100 g, as used by Boyd (1959d) in albino rats. In guinea pigs the $LD_{50} \pm SE$ was 1.33 ± 0.040 g per kilogram; in albino rats 1.42 ± 0.01 g per kilogram. The $LD_{50} \pm SE$ estimated from values for the three ingredients in the same manner as for albino rats was calculated to be 1.47 ± 0.071 g per kilogram. The LD_{50} found for the APC mixture was some 10% lower ($P < 0.05$) than was estimated from the sum of proportionate LD_{50}'s of the ingredients.

An estimate of the clinical signs was made in a similar manner and compared with values actually found upon the APC mixture. The comparisons are given in Tables 1 and 2. It may be seen that the intake of water during the second to fourth days was lower than estimated from the three ingredients. Ataxia occurred very commonly in phenacetin intoxication, rarely with the APC mixture. There was less hypothermia, inactivity or withdrawal, ataxia, and shivering, and more restlessness from the APC mixture than was estimated from the values found upon the three ingredients. Thus the syndrome of intoxication of the APC mixture is a composite of that of its ingredients with the signs of intoxication from acetylsalicylic acid tending to dominate the picture.

The causes of death from the APC mixture were similar to those noted in albino rats by Boyd (1959d) except that terminal hyperpyrexia was not found in guinea pigs. The histopathologic picture (Table 3) was similar to that recorded in albino rats.

DISCUSSION

The median lethal dose of acetylsalicylic acid in the guinea pig has not been previously reported (Smith, 1958). It was found during this investigation to be 29% higher ($P < 0.001$) than in the albino rat (Boyd, 1959a). The guinea pig is therefore no more sensitive than the rat to the acutely toxic effects of oral acetylsalicylic acid. This is in contrast to the sensitivity of the guinea pig to the toxic effects of certain other drugs such as penicillin (e.g., Schneierson and Perlman, 1956) and chlortetracycline (Ambrus *et al.*, 1952).

The syndrome of intoxication to acetylsalicylic acid in the guinea pig was similar to that in the albino rat (Boyd, 1959a). Most of the animals died within 12 hours from tonic-clonic convulsions associated with an acute gastroenteritis. Lish *et al.* (1959) have suggested that gastric irritation produced by acetylsalicylic acid may be associated with its ability to inhibit carbonic anhydrase. It would seem likely that such enzyme inhibition was only part of the mechanism of action since there was considerable necrosis of the gastrointestinal mucosa in the guinea pigs. When death was delayed beyond 48 hours, it was accompanied in addition by evidence of edema and congestion of the liver, lungs, and adrenal glands, and necrosis of the renal tubules.

Phenacetin in doses of the range of the oral LD_{50} produced hypothermia, stupor, and respiratory failure, mostly within 48 hours, associated with an acute gastroenteritis, edema, and/or congestion of the lungs, adrenals, pancreas, and to a lesser extent, the renal tubules. The LD_{50} did not differ significantly from the corresponding value in albino rats (Boyd, 1959b).

Excessive use of phenacetin has occurred in Alemanic Switzerland since World War II and has been suggested as a cause of a corresponding increase in the incidence of chronic interstitial nephritis (Gsell, 1958). Gsell *et al.* (1958) listed amongst the symptoms, lassitude, thirst, loss of weight, and vomiting; these occur in acute phenacetin poisoning as noted herein and by Boyd (1959b). Scheidegger (1958) listed the pathological findings as a shrunken kidney with proliferation of connective tissue, round cell infiltration of the cortex, and calcification of the papillae. Thölen (1958) concluded that phenacetin abuse was a probable cause; Pletscher (1958) was less certain.

Studer and Zbinden (1955) were unable to reproduce the renal lesions in rats given phenacetin in a dose of 500 mg per kilogram per day orally for over six months. Studer *et al.* (1958b) found that Saridon (which

contains phenacetin) did not augment renal lesions produced in rats and rabbits by various chemical nephrotoxic agents. Studer *et al.* (1958a) found that Saridon augmented an interstitial nephritis produced by intravenous injection of *Staphylococcus aureus haemolyticus* in rats. Phenacetin was found in this study and that of Boyd (1959b) to be able to produce toxic effects on the renal tubules in acutely lethal doses. While these lesions did not appear greater than those seen after acutely lethal doses of drugs such as benzylpenicillin ammonium (Boyd *et al.*, 1959), it is possible, though not necessarily so, that repeated daily use of phenacetin might have adverse effects on the kidneys.

The median lethal dose of caffeine was found to be some 20% higher ($P = 0.01$) in the guinea pig than in the albino rat (Boyd, 1959c). The syndrome, consisting of excitement, convulsions, gastroenteritis, and edema and/or congestion of the liver, heart, lungs, spleen, and adrenal glands, was similar to that found in albino rats by Boyd (1959c).

Addition of signs of toxicity of the three ingredients in proportions in which they were present in the APC mixture yielded, in most instances, figures which were insignificantly different from the corresponding figure obtained by use of the APC mixture directly. On the basis of data upon the median lethal dose, the toxicity of the APC mixture was 10% greater in guinea pigs, 15% less in albino rats (Boyd, 1959d), than the sum of the toxicity of the three ingredients. The central nervous system was stimulated by median lethal doses of acetylsalicylic acid and depressed, through hypothermia, by corresponding doses of phenacetin. The effect of the APC mixture upon the central nervous system was more akin to that of acetylsalicylic acid than that of phenacetin. The APC mixture and its three ingredients produced a gastroenteritis and toxic effects of varying degree upon various organs of the body.

SUMMARY

The clinical and pathological syndrome of intoxication from median lethal doses of a mixture of acetylsalicylic acid, phenacetin, and caffeine, given orally to guinea pigs, was found to be, in general, similar to a summation of the corresponding syndromes of the three ingredients given separately. Values for the $LD_{50} \pm SE$ of the three ingredients and of the mixture were, respectively, 1.19 ± 0.073, 1.87 ± 0.074, 0.230 ± 0.021, and 1.33 ± 0.040 g per kilogram body weight. At these doses, all four agents produced a gastroenteritis and toxic effects of varying degree upon several organs. Acetylsalicylic acid and caffeine excited the central nervous system, phenacetin depressed through marked hypothermia, and the mixture usually excited.

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THE CHRONIC ORAL TOXICITY OF CAFFEINE[1]

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Abstract

The toxicity of caffeine administered daily by intragastric cannula to female albino rats was determined over 100 days, i.e., 1/10 of the animal's life-span. The maximal $LD_{0(0.1L)}$ or maximal daily dose producing no deaths over 100 days was 110 ± 2.5 mg/kg; the $LD_{50(0.1L)}$, 150 ± 3.1 mg/kg; and the minimal $LD_{100(0.1L)}$, 191 ± 5.7 mg/kg. Doses of the order of the $LD_{0(0.1L)}$ produced a mild cerebral hyperemia, occasional psychotic-like self mutilation, gastric ulcers, and inhibition of oogenesis; hypertrophy of the salivary glands, gastrointestinal tract, liver, heart, kidneys, and lungs; a stressor reaction in the adrenal and thymus glands; minor changes in organ water levels; an occasional death apparently from bronchopneumonia; but in general a normal appearance, and no change in growth rate, eating and drinking habits, or in the volume, pH, and protein and glucose content of the urine. Doses of the order of the $LD_{50(0.1L)}$ also produced a polydipsia and diuresis and an occasional dermatitis; some degree of toxic nephritis, hepatitis, myositis, thyroiditis, and loss of red pulp in the spleen. Doses of the order of the minimal $LD_{100(0.1L)}$ and higher produced an increasingly more toxic reaction, which was similar to that seen in the syndrome at the acute oral LD_{50} dose and that due to fortnight partial starvation. Abrupt withdrawal of caffeine was followed for 1 week by decreased locomotor activity, a slight decrease in colonic temperature, and proteinuria and glycosuria.

Introduction

This project was undertaken to obtain information on the chronic toxicity of caffeine, a drug which is widely used not only in beverages such as tea, coffee, and various soft drinks, but also combined with agents such as acetylsalicylic acid and phenacetin in analgesic mixtures available without prescription.

From information in the literature it may be estimated that the daily administration of caffeine, usually in the diet or drinking water, in doses less than 100 mg/kg per day produces no or minimal signs of toxicity in rats (1, 2), rabbits (3), and dogs (3). At daily doses which approximate 100 mg/kg, sublethal toxicity has been reported in dogs (4), an occasional death in rats (5), and death after 11 to 16 days in rabbits (3). Larger daily doses have generally produced death in all species studied.

The LD_{50} ± S.E. of caffeine administered by intragastric cannula is 192 ± 18 mg/kg in rats (6) and 230 ± 21 mg/kg in guinea pigs (7). Doses of this order produce vomiting in dogs and man (6) but if vomiting does not occur or if tolerance to the emetic action develops (3), death may follow.

Methods

The experiments were performed upon female CBL Wistar albino rats of 150 to 200 g body weight, fed Purina laboratory chow and water ad libitum.

[1]This project was supported by a grant from the Medical Research Council of Canada.

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The acute oral $LD_{50} \pm$ S.E. of caffeine administered in the morning to rats which had not been starved overnight to empty the stomach was determined in 50 animals given doses of 200 to 350 mg/kg, and observed for 5 days. Caffeine (Merck Reagent) was dissolved in distilled water and given intragastrically in a volume to supply 20 ml/kg, each dose to 10 rats. Each rat was housed separately in a metabolism cage.

Caffeine was then administered daily in the morning, 5 days a week, in a series of decreasing fractions of the acute oral LD_{50} determined in unstarved rats. When caffeine was given for 1 week or longer, the dose was multiplied by 5/7 to determine the average daily dose per week. Each daily dose was given until 60% of the animals had died or for 100 days (1/10 the life span of a rat), whichever occurred first. Each daily dose was selected from results obtained with the previous dose, beginning with a large dose which produced deaths in a few days. The maximal $LD_{0(0.1L)}$ was the maximal daily dose which had killed no rats over this period, the $LD_{50(0.1L)}$ the daily dose which had killed 50% of the animals, and the minimal $LD_{100(0.1L)}$ the minimal daily dose which had killed 100% of the rats at 100 days. In order of selection, the daily doses were 264, 238, 181, 165, 158, 142, and 136 mg/kg, with 20 rats receiving each dose, and, for each dose of caffeine, 8 to 12 controls were given daily the same volume of distilled water (see Table I). Pair-fed controls were not introduced since pilot experiments indicated that food intake was not reduced except premortally.

Body weight was initially the same in all dosage groups. Older and heavier females are more susceptible to death from caffeine than are younger animals of lower body weight (8, 9), a fact confirmed in our laboratory (10).

Clinical measurements were made upon each rat in its metabolism cage on the day before, and at weekly intervals (or shorter if indicated) after the beginning of the administration of caffeine. The measurements made were body weight in grams, food consumption in grams chow per kilogram body weight per 24 hours, water consumption in milliliters per kilogram per 24 hours, colonic temperature in degrees Fahrenheit, urinary volume in milliliters per kilogram per 24 hours, urinary glucose in milligrams per kilogram per 24 hours, urinary protein in milligrams per kilogram per 24 hours, urinary pH, and other clinical signs as they appeared, quantitated as 1+ to 4+. Premortal signs were recorded in detail when possible. Colonic temperature was recorded by a Thermistemp Tele-Thermometer with the probe inserted 8 to 10 cm into the rectum. Urinary glucose, protein, and pH were measured with Ames Combistix reagent strips.

Autopsies were performed upon survivors at 100 days or after 60% of the animals had died, whichever occurred first. The animals were killed with chloroform and immediately autopsied to avoid postmortem shifts in fresh weight and water levels (11). Gross and microscopic appearance, fresh wet weight, and water levels were recorded on the organs listed in Table II. Histopathological examinations were made upon blocks of tissue fixed in Lillie's

buffered formalin and stained with hematoxylin-phloxine-saffron. Wet weight was measured in grams. The sample of skeletal muscle was the left half of the ventral abdominal wall muscles. The contents of the lumen of the gastrointestinal organs were removed by a standardized washing and milking procedure before weighing. Water levels were measured by drying a weighed aliquot of fresh tissue to constant weight at 95 °C in a Fisher Forced-Draft Isotemp oven. The sample of skin for water analysis was from the dorsolumbar region. Residual carcass was cut into small pieces, homogenized in a Waring blendor, and an aliquot selected for measurement of water level. Water levels were calculated as grams water per 100 g dry weight.

Sufficient animals given 136 mg/kg per day were alive at 100 days to permit a pilot study of the effects of sudden withdrawal of caffeine in six experimental rats and five controls. In addition to the measurements noted above, locomotor activity was determined by placing each rat in a Wahmann activity drum for 24 hours every 3rd day for 2 weeks before and 3 weeks after withdrawal of caffeine. At the end of 3 weeks after withdrawal, the animals were autopsied.

Except as noted below, the results were analyzed by t testing the differences between means in the caffeine-treated and control groups, and by plotting these differences against the daily doses of caffeine given, as noted above, for 100 days or until 60% of the animals had died. Statistical methods were those of Croxton (12), Waugh (13), and Goulden (14).

Results

The acute oral $LD_{50} \pm$ S.E. for caffeine given orally to unstarved rats was estimated (15) to be 264 ± 10 mg/kg.

In rats given caffeine daily, the dose killing 50% of animals was calculated from dose–response regressions at weekly intervals. These values are plotted against their interval of estimation in Fig. 1. The $LD_{50(0.1L)}$, or daily dose killing 50% of animals when administered for 100 days or 1/10 the rat's life span, was 150 ± 3.1 mg/kg per day.

Corresponding regression lines of the minimal LD_{100} and maximal LD_0 are plotted against days on a log scale in Fig. 2. The minimal $LD_{100(0.1L)}$ was calculated to be 191 ± 5.7 mg/kg per day and the maximal $LD_{0(0.1L)}$, 110 ± 2.5 mg/kg per day.

Growth was inhibited only in animals given daily doses in excess of the $LD_{50(0.1L)}$. The incidence of inhibition of growth was calculated from the incidence of animals with weekly growth rates less than the mean growth rate of the controls in the drug-treated (X_d) and control (X_c) groups, the incidence of X_c usually being 0.5 N/N or 0.5. The percentage incidence of growth inhibition was then calculated as $((X_d - X_c)/X_c) \times 100$ and was found to be dose-dependent. The average of weekly percentage incidences of growth inhibition was calculated over the duration of administration of each daily dose of caffeine, and these averages were plotted against daily dose. The estimating equation of the regression line for the scatterplot was found to be

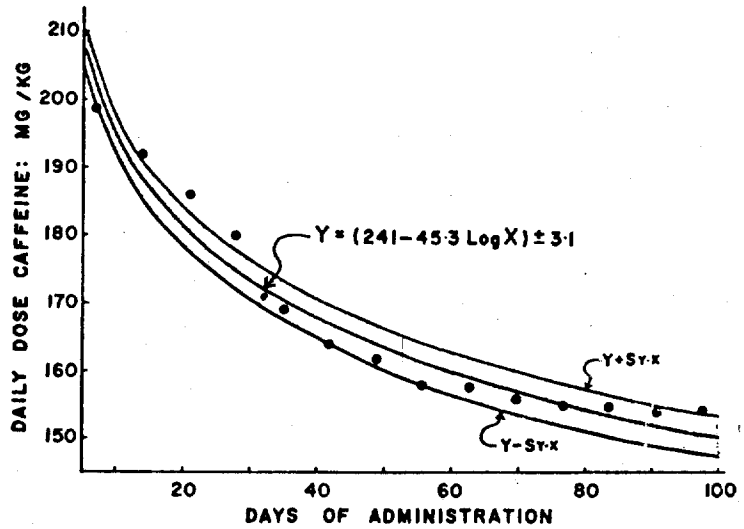

FIG. 1. The regression, on days of administration, of daily doses of caffeine which killed 50% of animals.

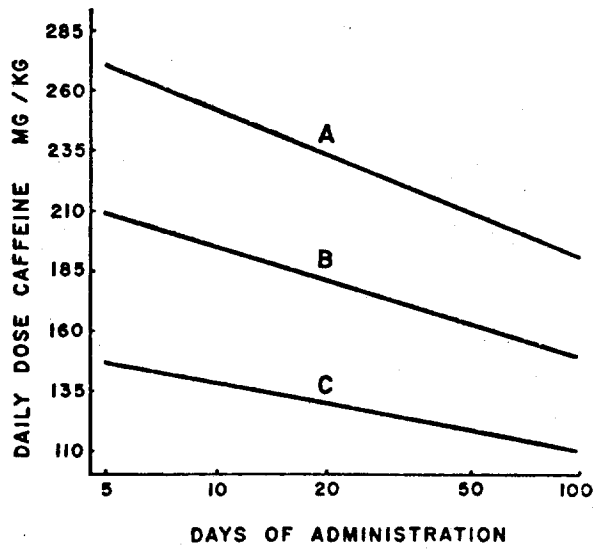

FIG. 2. The regression, on days of administration on log scale, of the minimal daily dose of caffeine producing 100% mortality (line A) and the maximal daily dose producing 0 mortality (line C). Line B is for the daily dose producing 50% mortality redrawn from Fig. 1 for comparison.

$Y = (299 \log X - 639) \pm 16$ and this line is shown in Fig. 3. The correlation coefficient of the regression was +0.943, and highly significant ($P < 0.001$).

Food intake was not significantly affected by any daily dose of caffeine except during the week before death, when it decreased. The regression on

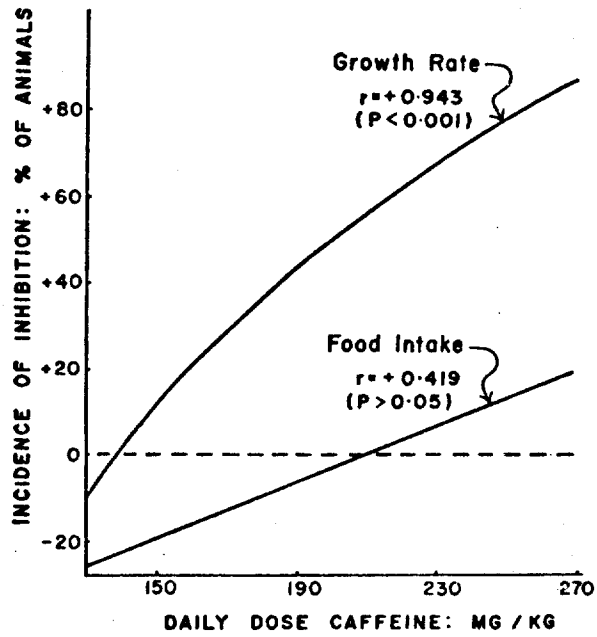

FIG. 3. The regression, on daily dose of caffeine, of the mean percentage incidence of inhibition of growth rate and of food intake.

daily dose of mean percentage incidence of inhibition of food intake, calculated as for inhibition of growth, is illustrated as a regression line in Fig. 3. The regression coefficient was not statistically significant. The tendency of the regression line to rise at the right in Fig. 3 is due to the higher death rate, and consequently a greater incidence of animals with premortal anorexia, following larger daily doses of caffeine. Representative values for food intake in the controls are listed in Table I.

Polydipsia occurred in all caffeine-treated animals, except in a few given the lowest daily dose. The polydipsic water intake was calculated by subtracting the mean water intake of control rats, exemplified in Table I, from that of caffeine-treated rats at each interval of measurement.

Polydipsic water intake did not vary significantly from week to week but it increased with increasing daily dose of caffeine. During the week before death, there was a decline in the extent of polydipsia. The regression on daily dose of mean polydipsic water intake was quadratic and could be fitted by the estimating equation $Y = 0.68X - 0.00017X^2 - 37.1$. The data were fitted better, however, by two equations for linear regression, one up to and a second beyond a daily dose of 238 mg/kg. Lines drawn from these linear equations are illustrated in Fig. 4. From this figure and the data given in Table I, it may be calculated that a daily dose of caffeine equal to the maximal $LD_{0(0.1L)}$ produced no polydipsia, one equal to the $LD_{50(0.1L)}$ produced a water intake

TABLE I

Body weight and measures of intake and output in 64 control female albino rats*

Measurement	Days							
	0	7	14	28	42	56	70	100
Body weight (g)	180 ± 13	186	192	203	213	217	225	232
Food intake (g/kg per day)	105 ± 21	101	120	106	92	83	82	80
Water intake (ml/kg per day)	159 ± 31	137	141	128	124	103	108	110
Colonic temperature (°F)	98.0 ± 0.9	98.0	98.3	98.4	99.2	99.0	98.1	99.2
Urine volume (ml/kg per day)	15.7 ± 8.7	9.7	8.7	4.9	3.0	2.9	3.4	3.9
Urine protein (mg/kg per day)	6.5 ± 4.7	7.4	7.4	7.8	4.7	2.5	4.1	7.2
Urine glucose (mg/kg per day)	5.4 ± 8.0	5.6	6.0	4.9	3.8	0.0	2.6	0.0
Urine pH	6.7 ± 0.5	6.8	6.9	6.9	6.5	6.5	6.5	6.4

*The results at day zero are expressed as mean ± standard deviation and at days 7 to 100 as mean only, the standard deviation averaging approximately the same percentage of the mean for each measurement at each interval.

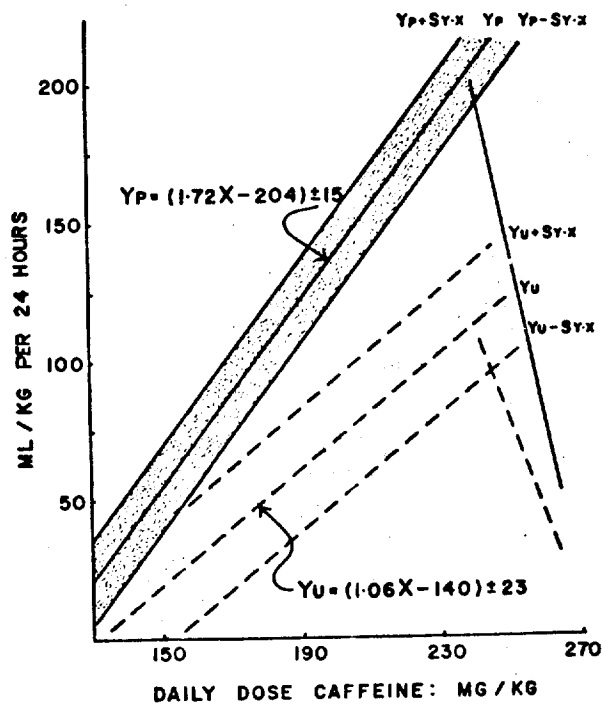

FIG. 4. The regression, on daily dose of caffeine, of mean values for polydipsic water intake (Yp and solid lines) and diuretic urinary volume (Yu and interrupted lines). The declining regression lines to the right are due to inclusion of numerous premortal declines in water intake.

some 40 to 50% greater than that in the controls, and one equal to the minimal $LD_{100(0.1L)}$ produced a water intake double that of the controls.

A diuresis occurred in all rats given caffeine in daily doses greater than the maximal $LD_{0(0.1L)}$. Regression lines for mean diuretic urinary volume, calculated in the same way as was mean polydipsic water intake, are indicated in Fig. 4. With data for control animals listed in Table I, it may be calculated that at the $LD_{50(0.1L)}$ dose urinary volume was increased by 4-fold, and at the minimal $LD_{100(0.1L)}$ by 16-fold. There was a premortal decline in the diuresis.

An augmented output (milligrams per kilogram per day) of urinary protein and of reducing substances calculated as glucose accompanied the diuresis from increasing daily doses of caffeine. Regression lines for these increases, calculated in the same way as diuretic urinary volume, are shown in Fig. 5. The correlation coefficients were +0.922 and +0.927 respectively and were highly significant. A considerable increase in the output of urinary protein and glucose occurred only at doses of the order of the minimal $LD_{100(0.1L)}$.

Corresponding regression lines for urinary pH and colonic temperature are included in Fig. 5. Neither of these parameters was significantly altered by caffeine administration except premortally.

Three other clinical signs appeared in some rats. During the first week of

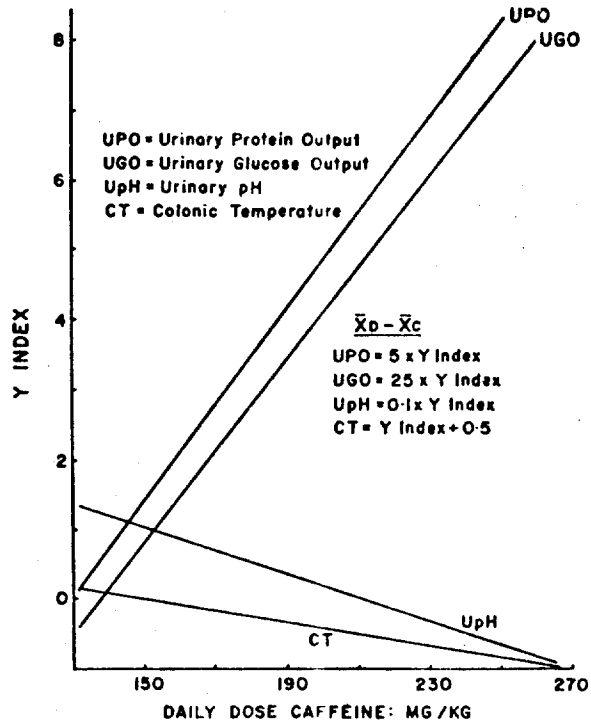

FIG. 5. The regression, on daily dose of caffeine, of mean changes in the output of urinary protein and glucose, and in the urinary pH and colonic temperature. The Y index is converted to appropriate units by use of the indicated factors.

caffeine administration, blepharitis occurred in a few animals at high dosage levels. Toward the end of 1 month's administration, a few rats had a psychotic-like reaction which took the form of the animal biting and mutilating its own feet or tail. During the third month, a few rats given intermediate doses developed alopecia and a dermatitis.

Premortal clinical signs in animals dying within a week from the larger daily doses of caffeine were convulsions and respiratory failure. When death occurred after the first week, it was preceded by anorexia, a relative oligodipsia and oliguria, loss of body weight, a fall in colonic temperature, proteinuria, aciduria, and often glycosuria.

The regressions on daily dose of caffeine of significant changes in the fresh weight of 14 organs plus residual carcass are shown in Fig. 6 as regression lines drawn from estimating equations. Doses of caffeine between the maximal $LD_{0(0.1L)}$ and $LD_{t0(0.1L)}$ produced a significant increase in the wet weight of lungs, adrenal glands, salivary glands, all parts of the gastrointestinal tract, liver, kidneys, and heart, and loss of weight in the thymus gland. As the daily dose of caffeine was increased there was progressively less increase in the

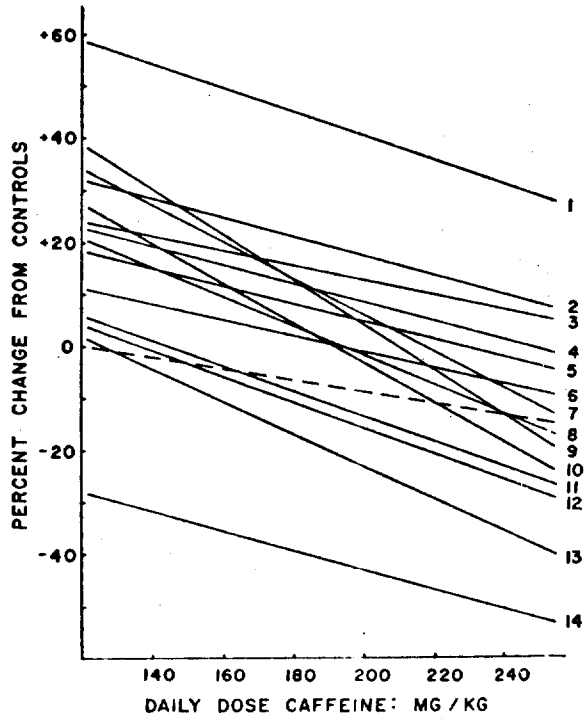

FIG. 6. The regression, on daily dose of caffeine, of significant percentage changes in the fresh wet weight of organs at autopsy. Percentage changes were calculated as $((\bar{X}_d - \bar{X}_c)/\bar{X}_c) \times 100$, where $\bar{X}_d$ is the mean in the drug-treated and $\bar{X}_c$ in the control animals. Legend: 1 = lungs; 2 = adrenal glands; 3 = heart; 4 = cardiac stomach; 5 = pyloric stomach; 6 = kidneys; 7 = submaxillary salivary glands; 8 = cecum; 9 = small bowel; 10 = liver; 11 = ovaries; 12 = skin; 13 = spleen; 14 = thymus gland. Changes in residual carcass are indicated by the interrupted line.

weights of the organs, and at doses above the minimal $LD_{100(0.1L)}$ some of them lost weight. None of the daily doses of caffeine significantly affected the wet weight of brain and thyroid gland. The regression of changes in the wet weight of skeletal muscle (anterior abdominal wall muscles) was quadratic: at daily doses of 136, 238, and 264 mg/kg there were no significant changes, but at doses from 158 to 181 mg/kg the muscle weight was significantly decreased by 14 to 23%.

Water levels were slightly increased in the gastrointestinal tract, ovaries, and skin of animals receiving doses less than the minimal $LD_{100(0.1L)}$. At larger daily doses they were also increased in adrenal glands, liver, muscle, and residual carcass. Correlation with daily dose was significant only in three organs, namely adrenal glands, pyloric stomach, and liver, and these correlations were positive. The results are summarized in Table II.

Histopathologic observations are summarized in Table III. Bronchopneumonia was the main finding in rats receiving caffeine in daily doses less than the

TABLE II

Effect of caffeine on water content of organs of rats[1]

	Daily dose of caffeine	
	Less than minimal $LD_{100(0.1L)}$ (N = 27 plus 28 controls)	$LD_{100(0.1L)}$ and greater (N = 11 plus 11 controls)
Adrenal glands	− 2.4	+24.3**
Brain	− 0.7	+ 1.0
Gastrointestinal tract:		
(*a*) Cardiac stomach	+ 5.9*	+ 3.2
(*b*) Pyloric stomach	+ 2.8	+14.6*
(*c*) Small bowel	+ 6.3*	+10.8*
(*d*) Cecum	+ 9.0*	+ 0.3
(*e*) Colon	+ 4.2	+ 3.0
Heart	+ 8.6	+ 3.4
Kidneys	+ 2.7	+ 0.2
Liver	+ 3.5	+12.9*
Lungs	+ 5.2	− 1.8
Muscle (abd. wall)	− 0.8	+ 9.0*
Ovaries	+11.1*	− 0.7
Salivary glands (submax.)	− 1.1	− 8.6
Skin	+26.4*	+18.6**
Spleen	+ 2.0	+ 4.3
Thymus gland	+11.1	− 1.2
Residual carcass	+10.8	+12.1*

[1]Water levels were measured as grams water per 100 g dry weight of tissue. The results are expressed as percentage change from controls, specifically as $((\bar{X}_d - \bar{X}_c)/\bar{X}_c) \times 100$, where $\bar{X}_d$ is the mean in the drug-treated rats and $\bar{X}_c$ the mean in the controls. Differences significant at $P = 0.05$ to 0.02 are indicated by one asterisk, and at $P = 0.01$ or less by two asterisks.

$LD_{50(0.1L)}$ and was apparently the immediate cause of death. Associated with the bronchopneumonia were parenchymal capillary congestion and edema, perivascular edema, and the frequent appearance of septal or "heart failure" cells in the lungs. Small ulcers extending about halfway down the glands of the pyloric stomach were occasionally seen. The dermatitis seen on gross examination was found to be associated with hyperemia and leucocytic infiltration of the skin. Increase in organ weights was found to be due to cortical hypertrophy in the adrenal glands and mucosal hypertrophy in the gut, and associated with hyperemia in the heart and mild vascular congestion in the kidneys. There was some inhibition of oogenesis in the ovary, a mild loss of red pulp in the spleen, a moderate loss of thymocytes in the thymus gland, and some deficiency of colloid in the thyroid gland. Increasingly larger daily doses of caffeine produced the increasingly greater toxic effects that are summarized in Table III.

Caffeine withdrawal was followed by a decrease in locomotor activity to half that before withdrawal and half that in the controls. The hypokinesia lasted 1 week, and was accompanied by a slight but significant fall in colonic temperature and an increase in the output of protein and glucose in the urine. There were no changes in the other parameters. Autopsy at 3 weeks after withdrawal revealed no significant changes in organ weights and water levels but small bronchopneumonic areas were found in the lungs.

TABLE III

The histopathological effects of caffeine on rats

Organ	Daily dose of caffeine (mg/kg)		
	136 and 142	158 and 165	181 and 238
Adrenal glands	Cortical hypertrophy	Cortical hypertrophy	Sinusoids congested
Brain	Mild hyperemia	Mild hyperemia	Meningeal congestion
Gastrointestinal tract			
(*a*) Cardiac stomach	Mucosal hypertrophy	Normal	Normal
(*b*) Pyloric stomach	Mucosal hypertrophy Occasional small ulcer	Capillary congestion Occasional small ulcer	Mild inflammation
(*c*) Small bowel	Mucosal hypertrophy	Mild hyperemia	Moderate inflammation
(*d*) Cecum	Mucosal hypertrophy	Mild hyperemia	Moderate inflammation
(*e*) Colon	Mucosal hypertrophy	Normal	Mild inflammation
Heart	Mild hyperemia	Mild capillary congestion	Marked capillary congestion
Kidneys	Mild edema and congestion	Edema and congestion, occasional venous thrombosis and thrombophlebitis	Marked edema and congestion
Liver	Normal	Mild sinusoidal congestion	Cloudy swelling and congestion
Lungs	Bronchopneumonia	Congestion, edema, thrombosis	Congestion, edema, hemorrhage
Muscle (abd. wall)	Normal	Weak cross striation	Weak cross striation
Ovaries	Deficiency primary follicles	Mild congestion	Mild congestion
Salivary gland (submax.)	Normal	Normal	Mild hyperemia
Skin	Normal	Occasional dermatitis	Normal
Spleen	Mild loss red pulp	Moderate loss red pulp	Marked loss red pulp
Thymus gland	Moderate atrophy	Marked atrophy	Marked atrophy
Thyroid gland	Colloid deficiency	Mild congestion	

Discussion

In 1962 the average consumption of coffee in the United States was 16 pounds per person (16) or approximately 4 mg/kg per day of caffeine. A cup of strong coffee may contain up to 150 mg of caffeine (17); 3 cups a day would average about 10 mg/kg, 10 cups 30 mg/kg, and 20 cups 60 mg/kg. This last dose is approximately half the maximal $LD_{0(0.1L)}$ determined in rats.

The toxicity of a mixture of acetylsalicylic acid, phenacetin, and caffeine has been found to approximate the sum of the toxicities of the three ingredients (7, 18). A sublethal dose of each drug given at the same time can produce death. It is possible, therefore, that a high daily intake of caffeine could lower the lethal dose of other drugs in a similar manner.

At daily doses slightly above the maximal $LD_{0(0.1L)}$, caffeine produced a mild cerebral hyperemia. Coffee drinkers have been found on occasions to exhibit less satisfactory nervous reactions than those who do not drink coffee (16). Rats given daily doses of caffeine slightly above the maximal $LD_{0(0.1L)}$ also exhibited a stressor reaction in the form of hypertrophy of the adrenal cortex and atrophy of the thymus gland. Some rats given this daily dose developed a psychotic-like self mutilation, an observation which has been recorded previously in rats given 100 mg/kg orally for 5 days (19).

Gastric ulcers were found in rats given daily doses of the order of the maximal $LD_{0(0.1L)}$. Actually smaller doses of caffeine have been found to produce gastric ulcers in cats (20) and rats (19).

Inhibition of oogenesis was seen in rats given caffeine in daily doses slightly above the maximal $LD_{0(0.1L)}$. At doses which may be estimated at approximately 100 mg/kg per day, Vacca (4) in 1926 reported inhibition of spermatogenesis in dogs. If the daily dose is reduced to 40 to 50 mg/kg, however, caffeine may be given for half a year with no effect upon the reproductive capacity of male or female albino rats (2).

A significant increase in weight of the salivary glands and all parts of the gastrointestinal tract and liver was produced by daily doses of caffeine of the order of the maximal $LD_{0(0.1L)}$. These doses also produced a slight mean increase in food intake and body weight but at the value of N used, the mean increase was not significant. The heart and kidneys were hypertrophied. A slight but insignificant mean loss of weight in skeletal muscle, skin, and residual carcass (bone, muscle, connective tissue) apparently prevented a significant gain in body weight. Caffeine fed in the diet to rats for 28 days at a daily dose of 100 mg/kg has been reported to inhibit slightly the growth rate (1). There was an increase in weight of the lungs associated with areas of bronchopneumonia, which appeared to be the cause of death in animals given doses somewhat above the maximal $LD_{0(0.1L)}$.

As the daily dose of caffeine was increased through the range of the $LD_{50(0.1L)}$ and minimal $LD_{100(0.1L)}$, the toxic reaction progressively assumed clinical and pathological signs characteristic of the reaction at the acute LD_{50} (6). These signs included anorexia and loss of body weight, hypothermia, blepharitis,

gastroenteritis, nephritis, hepatitis, pulmonary edema, and congestion of the adrenal glands, brain, heart, ovaries, and thymus gland. The neurological signs, diarrhea, and generalized dehydration of acute toxicity (6) were not seen in the chronic studies, and convulsions were produced only by the top daily doses. The polydipsia and diuresis seen in survivors of acutely lethal doses (6) occurred in the chronic studies as the daily dose increased through the range of the $LD_{50(0.1L)}$ and minimal $LD_{100(0.1L)}$. These doses produced increasing glycosuria, confirming a report in 1912 by Salant and Rieger (3), and proteinuria; but the central lobular hepatic fatty degeneration reported by Stieve (21) in pregnant rabbits was not seen. Anorexia accompanying high daily doses produced the loss of organ weights and increase of organ water levels found in rats following fortnight partial starvation by Peters and Boyd (22).

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THE METABOLISM OF THE METHYLATED PURINES

II. URIC ACID EXCRETION FOLLOWING THE INGESTION OF CAFFEINE, THEOPHYLLINE, AND THEOBROMINE

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The wide-spread use of coffee, tea, and cocoa as beverages and their marked stimulatory and diuretic action have led to numerous investigations concerning the fate of caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), and theobromine (3,7-dimethylxanthine) in the animal body. Although it is generally accepted that these compounds are readily absorbed after oral or parenteral administration, only small amounts of the drugs are excreted unchanged (1–3). The isolation of the monomethylxanthines from the urine of both humans and experimental animals (2, 4–6) after the administration of the above compounds definitely showed that the di- and trimethylxanthines are at least partially demethylated. Krüger and his associate (7) suggested that in man the methyl group in position 3 is the most labile, while that in position 7 is the most stable, and that the mono- and dimethylxanthines normally found in human urine can be accounted for by the removal of the methyl group occupying position 3 from the methylated xanthines of the food.

With the partial demethylation of the di- and trimethylxanthines thus demonstrated, the question arose as to the possibility of a conversion of a portion of these compounds to uric acid by means of complete demethylation and an oxidation in the 8 position. Much of the earlier work concerning such a conversion of the methylated xanthines to uric acid is contradictory and open to criticism because of the use of dogs and rabbits as experimental animals. Both of these species excrete allantoin, and not uric acid, as the chief end-product of purine metabolism. However, most of the early work on human subjects, in which the analyses were made by the inaccurate gravimetric and titrimetric methods for uric acid then available, indicated that there was no increase in the urinary excretion of uric acid after the ingestion of caffeine, theophylline, or theobromine (8–10).

The development of colorimetric procedures for the determination of uric acid by Folin and his associates (11) and Benedict and Hitchcock (12)

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resulted in the reinvestigation of the problem and the formulation of the opposite point of view; *i.e.*, that the methylated xanthines *do* give rise to an increased excretion of uric acid in man. In 1916, Benedict (13), using the new ammoniacal silver precipitation procedure of Benedict and Hitchcock (12), reported an experiment in which the ingestion of 1 gm. of caffeine per day for 4 days led to an increased output of uric acid amounting to 100 to 160 mg. per day above control values. A short time later, Mendel and Wardell (14), using the same colorimetric method, reported that the ingestion of coffee, tea, or caffeine by their subjects was followed by an increased uric acid excretion. This increased excretion appeared to be proportional to the quantity of caffeine ingested and was equivalent to the amount of uric acid which would be obtained by the demethylation and subsequent oxidation of from 10 to 15 per cent of the ingested caffeine. Clark and de Lorimier (15) in 1926 studied both the blood levels of uric acid and the urinary excretion of uric acid after the ingestion of caffeine and theobromine. Both compounds caused an increase in blood uric acid levels as measured by Benedict's direct method (16). Caffeine administration was followed by a rise in uric acid excretion, as determined by the direct method of Benedict and Franke (17), while theobromine appeared to diminish the rate of excretion of uric acid. They concluded that the methylxanthines are not oxidized directly and that the increased uric acid excretion after caffeine ingestion is probably due to a stimulation of general metabolism.

Myers and Wardell (18) found that the ingestion of caffeine and theophylline by human subjects resulted in an increased uric acid excretion, as determined by both the direct colorimetric method of Benedict and Franke (17) and the silver isolation procedure of Benedict and Hitchcock (12). They confirmed the report of Clark and de Lorimier that theobromine causes no such increase. Myers and Wardell made the suggestion that a portion of the methylated xanthines might be oxidized in the 8 position before being completely demethylated, thus giving rise to methyluric acids. It was pointed out that the oxidation of theophylline (1,3-dimethylxanthine) in position 8 would give rise to 1,3-dimethyluric acid, while both oxidation and removal of the more labile methyl group in the 3 position would result in the formation of 1-methyluric acid. Both of these methylated derivatives of uric acid give the color reaction with the uric acid reagents. On the other hand, by a similar mechanism, theobromine (3,7-dimethylxanthine) would give rise to 3,7-dimethyluric acid or to 7-methyluric acid. Neither of these latter compounds causes any appreciable reduction of phosphotungstic acid. The formation of methylated uric acids by such an oxidation and demethylation mechanism was offered as a possible explanation for the differences observed in "uric acid" excretion following the administration of theophylline and theobromine.

Myers and Hanzal (19) later studied the metabolism of the methylated xanthines as well as the methylated uric acids in the Dalmatian dog. The oral administration of either caffeine or theophylline resulted in an increased excretion of uric acid as determined by colorimetric methods. Theobromine ingestion had little effect on the uric acid excretion. After the administration of theophylline, materials having the reducing properties of uric acid were isolated by precipitation with silver salts. After the removal of the silver and recrystallization, crystals resembling those of 1,3-dimethyluric acid were obtained. Similar studies made after the ingestion of theobromine suggested the formation of 3,7-dimethyluric acid.

These authors found that the methyluric acids which have a methyl group in position 7 give only traces of color with the phosphotungstic acid reagents. No increase in uric acid was observed after the ingestion of this group of compounds by the Dalmatian dog. Those methylated uric acids which *do not* have a methyl group attached in the 7 position all reduce phosphotungstic acid. The oral administration of this group of methylated uric acids to the Dalmatian hound caused an increase in uric acid excretion. However, the extra color observed could be ascribed to the elimination of the unchanged methyluric acids. In other words, the extra color was proportional to that which would be anticipated if the methyluric acids were excreted unchanged. From these results the workers concluded that the methylated derivatives of uric acid are "apparently quite stable in the animal organism."

Thus, the work of Myers *et al.* suggests that the increase in uric acid excretion observed after the administration of caffeine and theophylline is probably not due to uric acid itself but rather to its methylated derivatives. Certain of the methyluric acids do resemble uric acid in many of their properties and would be included as uric acid by both the direct and silver precipitation procedures. Consequently it is impossible by either of these methods of analysis to decide whether the extra uric acid is actually uric acid or whether it is due to the formation and excretion of the methylated uric acids. In order to investigate this problem further, an enzymatic procedure (20) has been developed for the specific determination of uric acid in the presence of its methylated derivatives. It is the purpose of this paper to report the application of this method to a study of the metabolism of the three methylated xanthines commonly found in the human diet.

EXPERIMENTAL

Metabolic studies were carried out on five different individuals to investigate the effect of the administration of caffeine, theophylline, and theobromine on uric acid excretion. In the first series of experiments, the general plan was to place the subject on a purine-free diet for a period of 9

days. This was divided into a 3 day control period, a 3 day experimental period during which 1 gm. per day of the methylxanthine in question was fed in two divided doses, and a final 3 day control period. Urine samples were collected over 24 hour periods and analyzed for total phosphotungstic acid-reducing materials, residual reducing materials, and true uric acid by the uricase procedure of Buchanan, Block, and Christman (20). Analyses were also made for uric acid by an adaptation of the ammoniacal silver precipitation method of Benedict and Hitchcock (12) to the Evelyn photoelectric colorimeter with the alkaline urea-cyanide reagent of Christman and Ravwitch (21) and the arsenophosphotungstic acid of Benedict (16, 17). Creatinine determinations were made on all 24 hour samples of urine to check on the completeness of the urine collection. Since these values showed only slight variations from day to day for a single individual, they have not been included in Tables I and II.

Table I contains a summary of the uric acid values for subject O. H. B. after the administration of caffeine, theophylline, and theobromine. This individual does not drink coffee or tea and this fact may explain the differences observed in the results for this subject as compared to those found for the two subjects (Table II) who are habitual drinkers of coffee.

The ingestion of caffeine caused an 84 per cent increase in the total reducing materials, from a 3 day average control value of 435 mg. per 24 hours to an average of 799 for the experimental days. The true uric acid increased only 5 per cent, from an average of 390 to 411 mg. per 24 hours. The striking feature of the experiment was the tremendous increase in residual color; *i.e.*, the phosphotungstic acid-reducing materials which are not oxidized by incubation with uricase. This residual material, measured in terms of uric acid, increased from an average value of 45 mg. per 24 hours for the 3 day control period to an average of 388 mg. per 24 hours for the experimental period, a 760 per cent increase. This rise in residual color is still evident on the day following the last administration of caffeine, probably because the last dose of drug was taken late in the preceding afternoon. It is because of this lag in the excretion of the residual material that the values obtained for the 3 days preceding the ingestion of the methylxanthines have been used in all cases to compute the average control values. In order to prove that sufficient uricase was being employed to destroy all of the uric acid in these experimental urines, known amounts of uric acid were added to these samples and shown to be completely oxidized by the enzyme.

Uric acid as determined by the silver precipitation procedure rose to a level averaging 95 per cent above that found for the control period. If the average for true uric acid for the experimental days (411 mg.) is subtracted from that for the silver precipitation (689 mg.) and the result

divided by the average increase in residual color (343 mg.), it is found that about 82 per cent of the extra non-uric acid reducing material is carried down in the silver precipitation fraction. The possibility that this residual

TABLE I

Effect of Ingestion of Methylated Xanthines on Uric Acid Excretion

Subject, O. H. B.; age, 28; weight, 130 pounds. All values are expressed as mg. excreted in 24 hours.

Date	Uric acid by uricase method			Uric acid by silver pptn.	Diet
	Total color*	Residual color*	True uric acid		
1943					
Mar. 29	412	42	370		Low purine
" 30	442	49	393	341	" "
" 31	450	43	407	375	" "
Apr. 1	719	302	417	621	1 gm. caffeine
" 2	889	454	435	780	1 " "
" 3	789	409	380	666	1 " "
" 4	497	114	383	439	Low purine
" 5	429	49	380	377	" "
" 6	370	40	330	290	" "
June 9	370	34	336	352	" "
" 10	454	44	410	419	" "
" 11	432	42	390	391	" "
" 12	850	454	396	549	1 gm. theophylline
" 13	897	509	388	590	1 " "
" 14	910	543	367	635	1 " "
" 15	537	212	325	366	Low purine
" 16	488	60	427	424	" "
" 17	415	61	354	364	" "
1944					
Mar. 9	406	34	372		" "
" 10	392	34	358		" "
" 11	358	34	324		" "
" 12	388	53	334	347	1 gm. theobromine
" 13	344	55	289	277	1 " "
" 14	401	57	344	351	1 " "
" 15	414	49	365		Low purine
" 16	444	55	389	396	" "

* Expressed in terms of uric acid.

color is due to the presence of methylated uric acids and the implications of such a hypothesis will be discussed below.

The ingestion of theophylline gave results similar to those obtained for caffeine. The average increases in total color and residual color were 112 and 1155 per cent respectively, while the slight increase in true uric acid

was within the experimental error of the method. The silver precipitation procedure showed an average increase of 52 per cent over the control values. By calculations similar to those described for caffeine, it was found that about 45 per cent of the extra non-uric acid reducing material is precipitated by the ammoniacal silver-magnesium mixture.

No significant changes in either total color or true uric acid were found after the administration of theobromine. Only a comparatively slight increase was observed in the residual color. The values obtained by the silver precipitation method agree very closely with those found for true uric acid by the uricase method. Two additional studies with theobromine on other subjects gave values confirming the results reported in Table I.

The effect of the ingestion of caffeine on the uric acid excretion of two other individuals and the results after theophylline dosage in one are shown in Table II. The results show that a considerable variation is found between individuals, which perhaps may be correlated with previous coffee drinking habits or age. The subjects of the experiments reported in Table II are habitual coffee drinkers, whereas the other subject (O. H. B., Table I) does not drink coffee or tea.

Subject A. A. C. showed a 20 per cent average increase in total color and a 187 per cent increase in residual color after the ingestion of caffeine. The true uric acid values decrease slightly but, again, this is within the experimental error of the method. Average values obtained by the silver precipitation procedure are approximately 13 per cent greater than those of the control period. About 80 per cent of the extra residual material is included in the silver-precipitable fraction. The addition of liver to the diet (high purine period in Table II) caused a prompt increase in true uric acid but had only a slight effect on the residual color.

The ingestion of caffeine by W. D. B. resulted in an increase of 227 per cent residual color, but had little effect on the true uric acid excretion. In this experiment the precipitation with silver salts appears to have carried down approximately 100 per cent of the extra residual material. The administration of theophylline caused a definite increase in residual material of 390 per cent on the 1st experimental day. The subject became ill from the effects of the drug on the 2nd day and all values show a marked decrease. However, the residual color was still considerably greater than on the control days. Calculations, based on data obtained for the 1st experimental day only, show that about 44 per cent of the extra residual material was precipitated by the ammoniacal silver reagent.

A summary of the values recorded in Tables I and II shows that the administration of caffeine and theophylline causes a marked increase in a non-uric acid, phosphotungstic acid-reducing material. The so called direct procedures include all of this reducing material as uric acid, while the

procedures involving a preliminary precipitation with silver salts appear to carry down with uric acid from 80 to 100 per cent of the extra reducing material excreted after caffeine ingestion and about 45 per cent of that excreted after theophylline administration. The uricase procedure, how-

TABLE II

Effect of Ingestion of Caffeine and Theophylline on Uric Acid Excretion

All values are expressed as mg. excreted in 24 hours.

	Date	Uric acid by uricase method			Uric acid by silver pptn.	Diet
		Total color*	Residual color*	True uric acid		
	May, 1943					
Subject, A. A. C.; age, 47; weight, 142 lbs.	11	615	69	545	577	Low purine
	12	469	54	415	421	" "
	13	515	56	459	480	" "
	14	628	159	469	541	1 gm. caffeine
	15	647	185	462	571	0.9 gm. caffeine
	16	564	131	433	480	Low purine
	17	565	71	494	514	" "
	18	550	67	482	485	" "
	19	692	79	613	653	High "
	June, 1943					
Subject, W. D. B.; age, 32; weight, 160 lbs.	2	431	75	356	399	Low "
	3	511	73	438	460	" "
	4	409	70	339	347	" "
	5	547	217	330	471	1 gm. caffeine
	6	665	295	370	584	1 " "
	7	601	206	395	542	1 " "
	8	577	104	473	520	Low purine
	9	734	63	661	677	" "
	10	658	66	592	610	" "
	11	534	62	472	470	" "
	12	584	83	501	497	" "
	13	661	341	320	439	1 gm. theophylline
	14	421†	190†	231†	291†	1 " "
	15	469	100	369	372	Low purine
	16	528	63	465	477	" "
	17	534	77	467	481	" "

* Expressed in terms of uric acid.

† The subject became ill from the effects of the drug.

ever, demonstrates that there is no increase in true uric acid after the administration of either caffeine or theophylline. Theobromine causes no significant change in uric acid excretion as measured by either the direct, the silver precipitation, or the uricase method.

The results of a study of the rate of excretion of uric acid and the non-uric acid reducing materials after the ingestion of caffeine and theophylline are shown in Fig. 1. Subject O. H. B. was placed on a purine-free diet for a period of 11 days. 500 mg. of caffeine were fed on the 2nd and 7th days and 500 mg. of theophylline on the 4th and 9th days. Analyses similar to those previously described were made on urines collected over 2, 3, and 6 hour periods, as indicated in Fig. 1. All values are calculated in mg. of material excreted per hour and are plotted against time in hours.

An hourly variation was observed in the amount of total phosphotungstic acid-reducing materials excreted during the control periods. The values

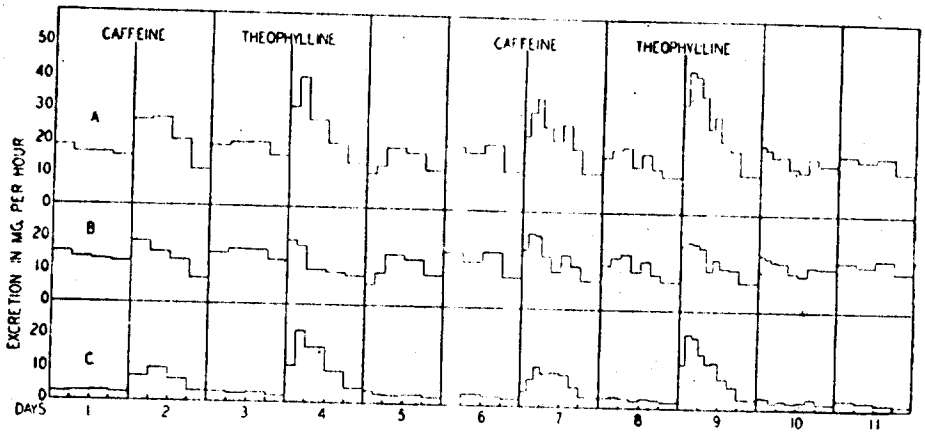

FIG. 1. Uric acid excretion following the ingestion of caffeine and theophylline by subject O. H. B. 500 mg. of caffeine were taken on the 2nd and 7th days and 500 mg. of theophylline on the 4th and 9th days. Each small division on the abscissa represents 6 hours. *A*, total phosphotungstic acid-reducing materials; *B*, true uric acid; *C*, non-uric acid reducing materials.

were usually higher from 8 a.m. to 2 p.m. and decreased during the early morning hours, but, in general, average from 15 to 18 mg. per hour. The ingestion of either caffeine or theophylline caused a definite increase in total color during the first 12 hours after administration, which was followed by a marked decrease during the second 12 hour period. It should be emphasized again that these values for total color are the sum of the two components, true uric acid and non-uric acid residual materials. Similar results would be observed if direct procedures for uric acid, such as the Benedict-Franke or Folin's direct method (22), had been used. Clarke and de Lorimier, using the Benedict-Franke method, obtained increases in uric acid excretion similar to those after the administration of caffeine.

Values for true uric acid ranged from 10 to 18 mg. per hour on the control days. The excretion was usually greater during the portion of the day

when the subject was more active and diminished during the early morning hours. The first ingestion of caffeine caused a slight increase in true uric acid during the first 6 hours, but this was followed by a gradual decrease to the very low level of 7.7 mg. per hour during the early morning hours. A similar increase and subsequent decrease were observed after the second administration of caffeine. Theophylline ingestion caused fluctuations in true uric acid resembling those found with caffeine. However, when the total amounts of true uric acid excreted over each 24 hour period are compared, it can be seen that the daily excretion of true uric acid is not significantly changed by the administration of either caffeine or theophylline. The initial increase in true uric acid excretion which occurs during the first 6 to 8 hours after the ingestion of these two methylated xanthines is compensated by a decreased excretion which occurs later in the day. It is suggested that this temporary increase in uric acid excretion is caused by either a stimulation in general metabolism or by the intense diuresis resulting from the ingestion of caffeine and theophylline in such large amounts. All subjects suffered very definite nervous stimulation after dosage with either drug and in the study reported in Fig. 1 the excretion of urine during the 2 hour period immediately following the administration of 500 mg. of theophylline averaged 8.8 cc. per minute as compared to 1 to 2 cc. per minute during the control periods.

The most striking results were those obtained for residual color, as shown in Fig. 1. While the hourly excretion during the control days was relatively constant and averaged from 2 to 4 mg. per hour, marked increases were observed after the ingestion of 500 mg. of caffeine or theophylline. The first administration of caffeine resulted in values for residual color of 7.5, 11.1, and 6.8 mg. per hour for each of the three 6 hour periods following dosage. The residual material excreted during the three 2 hour periods immediately following the second ingestion of caffeine amounted to 4.9, 8.4, and 12.2 mg. per hour, respectively. Theophylline produced an even greater rise in non-uric acid reducing materials, as shown by values of 22 mg. per hour for the 3 to 6 hour period following the first dosage, and 23 mg. per hour during the 2 to 4 hour period after the second administration. It can be seen from Fig. 1 that theophylline causes a much greater increase in residual materials and is more prompt in its action than caffeine.

Table III gives the total 24 hour values calculated from the data presented in Fig. 1. The figures listed in the third column show the remarkably consistent values obtained for the non-uric acid reducing materials excreted by an individual on a low purine diet for a number of days, and the very definite increases in this fraction after the ingestion of caffeine and theophylline. The values of 173 and 179 mg. per 24 hours after the ingestion of 500 mg. of caffeine and 283 and 272 mg. after the administration

of 500 mg. of theophylline show that the ingestion of a certain amount of methylxanthine causes the excretion of a definite, consistent amount of non-uric acid reducing material by any one individual. From the results given in the fourth column, it can be seen that the excretion of true uric acid is not significantly increased by the administration of either caffeine or theophylline when considered over 24 hour periods.

TABLE III

*Effect of Ingestion of Caffeine and Theophylline on Uric Acid Excretion**

Subject, O.H.B.; age, 29; weight, 130 pounds. All values are expressed as mg. excreted in 24 hours.

Date	Total reducing materials†	Non-uric acid reducing materials†	True uric acid	Diet
Apr., 1944				
19	397	63	334	Low purine
20	509	173	336	500 mg. caffeine
21	442	63	379	Low purine
22	576	283	293	500 mg. theophylline
23	357	67	290	Low purine
24	415	68	347	" "
25	532	179	353	500 mg. caffeine
26	375	66	309	Low purine
27	626	272	354	500 mg. theophylline
28	399	62	337	Low purine
29	395	49	346	" "

* Data from Fig. 1 summarized for 24 hour periods.

† Measured in terms of uric acid.

DISCUSSION

The oral administration of caffeine, theophylline, or theobromine has little effect on the excretion of true uric acid over 24 hour periods, as determined by the uricase procedure. However, the ingestion of caffeine and theophylline does cause the excretion of considerable amounts of a phosphotungstic acid-reducing material which is not oxidized by the enzyme uricase. All of this non-uric acid reducing material is included as uric acid by the direct colorimetric methods for the determination of uric acid, and a portion of the material is carried down with uric acid by the indirect colorimetric procedures involving a preliminary precipitation with silver salts. This non-specificity of the older colorimetric procedures for determining uric acid thus would seem to be the explanation for the numerous reports in the literature of an increased uric acid excretion following the administration of these two methylated xanthines.

Myers and coworkers were the first to suggest the possible formation of

methylated uric acids by the oxidation of the methylxanthines. They further showed that certain of the methyluric acids reduce phosphotungstic acid and are precipitated by both ammoniacal silver and acid silver lactate. However, they published no proof of their hypothesis and had no analytical procedure available to distinguish between uric acid and its methylated derivatives. The results obtained by the application of the more specific enzymatic procedure to a study of the problem show that the extra reducing material excreted after the ingestion of caffeine and theophylline is not uric acid, but this method still offers no direct proof as to the identity of the non-uric acid materials.

Ascorbic acid, resorcinol, ergothioneine, glutathione, cystine, and several of the methyluric acids reduce phosphotungstic acid under the alkaline conditions used in the uric acid determinations, and none of these substances is oxidized by incubation with uricase (20). Urine samples collected before and after the administration of caffeine to one of the subjects in the present study were analyzed for ascorbic acid, but no increases were observed. Glutathione and cystine give only 0.51 and 0.54 per cent as much color with phosphotungstic acid as uric acid (20). Therefore nearly 300 gm. of these substances would have to be excreted over a 4 day period to account for the 1500 mg. of extra residual material excreted by O. H. B. after the ingestion of 3 gm. of theophylline. Since large proportions of the residual material are precipitated by ammoniacal silver, resorcinol could not be a source of the residual color. Ergothioneine has a chromogenic value 24 per cent of that of uric acid and is precipitated by ammoniacal silver. Analyses for ergothioneine were not carried out, but the excretion of 6 gm. of this material in 4 days would be required to account for the 1500 mg. of residual material mentioned above.

The methyluric acid hypothesis suggested by Myers and coworkers appears to be the most probable explanation for the increases in residual color. Only three of the methylated uric acids reduce phosphotungstic acid to any appreciable degree (20). Under the conditions of the uricase procedure 1-methyluric acid, 1,3-dimethyluric acid, and 3-methyluric acid give an amount of color equivalent to 105.1, 50.9, and 31.9 per cent respectively of that given by uric acid. Of these three methyluric acids, only the 1-methyluric acid is precipitated by ammeniacal silver under the conditions used throughout this work. It is not precipitated from weak lithium carbonate solutions by ammoniacal silver, but when added to urine is precipitated almost completely along with uric acid.

By the mechanism suggested by Myers *et al.*, caffeine could give rise to all of the tri-, di-, and monomethyluric acids which have methyl groups substituted in the 1, 3, or 7 position. Either the 1,3-dimethyluric acid, 1-methyluric acid, or 3-methyluric acid would have to be formed to account

for the reduction of the color reagents. It would appear, then, that at least a portion of the caffeine must be demethylated in the 7 position.

For subjects O. H. B. and A. A. C. about 80 per cent of the extra residual material was precipitated by ammoniacal silver, while nearly 100 per cent was carried down in the experiment on W. D. B. Only 1-methyluric acid is precipitated by this procedure. Therefore, it is suggested that from 80 to 100 per cent of the extra reducing material excreted after caffeine ingestion is 1-methyluric acid, while the remainder is either 1,3-dimethyluric acid, 3-methyluric acid, or a mixture of both compounds. The absolute amount of these compounds which could be formed appears to be subject to individual variations.

1,3-Dimethyluric acid would be formed by the oxidation of theophylline in position 8, while either 1-methyluric acid or 3-methyluric acid would result from a combination of oxidation and partial demethylation. From the results listed in Table I, subject O. H. B. appears to have converted about 50 per cent of the 3 gm. of theophylline ingested into chromogenic methyluric acids. About 45 per cent of the latter was precipitated by ammoniacal silver and therefore would seem to be 1-methyluric acid.

By a simple oxidation in position 8, theobromine would be converted into 3,7-dimethyluric acid. An oxidation and partial demethylation would result in the formation of either 3-methyluric acid or 7-methyluric acid. Of these three possible metabolites only 3-methyluric acid reduces phosphotungstic acid to any appreciable extent. Therefore, the only conclusion which can be made from the present study concerning the metabolism of theobromine is that neither uric acid nor 3-methyluric acid is formed in any significant amount.

The authors appreciate that the final proof that the non-uric acid reducing materials are methyluric acids awaits the isolation of these compounds. After the present metabolic experiments were completed, preliminary work on such isolations was started. Since the entire time of two of the authors was directed to a war project, it was necessary that this work be temporarily postponed.

SUMMARY

The ingestion of caffeine or theophylline by human subjects is followed by a definite increase in the excretion of phosphotungstic acid-reducing materials as measured colorimetrically by both the direct or silver precipitation methods for the determination of uric acid. The administration of theobromine causes no such increase in uric acid excretion.

The fractionation of this chromogenic material into true uric acid and residual reducing materials by the use of the enzyme uricase shows that the true uric acid excretion is increased very little, if at all, over a 24 hour period.

A slight increase in true uric acid does occur during the first 6 hours after the administration of caffeine or theophylline, but a diminished output later in the day compensates for this temporary increase. It is suggested that this slight increase is due to diuresis or a temporary stimulation of metabolism rather than to a conversion of the methylxanthines to uric acid.

The ingestion of caffeine or theophylline causes a very definite increase in the excretion of phosphotungstic acid-reducing materials which are not oxidized by incubation with uricase. Evidence has been presented which suggests that these materials may be 1-methyluric acid, 3-methyluric acid, or 1,3-dimethyluric acid formed by the oxidation and partial demethylation of the methylated xanthines.

The authors wish to express their appreciation to Miss Naomi Geib for assistance in making the analyses reported in Fig. 1.

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The Importance of Coffee on the Human Organism

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At the present time, coffee has become one of the most frequently used foods, although the history of its use by man in Europe - as opposed to alcohol and tobacco - scarcely exceeds 400 years. In the research of the influence of coffee on the human organism, a turn was produced in 1820 when Runge discovered caffein as the principle in coffee, and in 1899, Fisher defined its structural formula. Since then, there are always discussions on the subject of the usefulness or the harmfulness of coffee for the human organism and, not a day goes by when the doctor is not called upon to answer questions of patients if they are allowed, during their illness, to have coffee or not. These problems are really important, because a doctor uninitiated in the effects of coffee and forbidding in an overall way the use of coffee will cause more harm than good. It is certain that coffee has many good qualities, that it produces a favorable effect on the human organism, but that it can be harmful, sometimes as the consequence of a false use. For this reason, one must know exactly all the effects produced on the organism.

In our report, we will have as our objective the effects which coffee produces on the organism of intellectual. This is the social class which most uses, perhaps every day, the beneficial effects of coffee. One can characterize the intellectual as a man exercising the most complicated intellectual activity with the predominant element of creative activity. This creative activity requires from the psychological viewpoint, a high degree of perception, concentration, abstraction and associating disposition. It is then natural that an intellectual looks for means to intensify the abilities mentioned above or to renew them, if he is tired as a result of exhausting intellectual activity and his psychic abilities are lowered.

An experiment of long years has shown that coffee possesses these required qualities.

It is believed at the time of the discovery of caffein in coffee that the active principle, perhaps even the only one, in coffee was justly caffein.

Today, we know that coffee is a complex food, containing besides caffein many diverse substances. Caffein is not found in coffee in a free form, but is rather fixed together with potassium and chlorogenic acid in the form of a complex substance. In addition to these components, coffee still contains caffeic acid, quinic

acid, trigonelline, coffee fat and choline and many others as are shown in Diagram 1.

The porportion of caffeine in roasted coffee is from 1.3 to 1.4 g percent, in the infusion of 100 g of coffee, 1.08 g percent, the rate of chlorogenic acid is 4.1 g percent, and 3.3 g percent in the infusion. As a result of the fixation of the caffeine, there is produced the slowest relief and its slowest resorption in the intestine; from this fact, the effect of coffee is slower and less intense than that of pure caffeine.

Perhaps the most important effect in intellectuals and the most appreciated is the effect carried out on the central nervous system. The substances contained in coffee - and not only caffeine, as will be seen below - exerts a stimulating effect on the cortex, on the thalamic vegetative centers, in the medulla oblongata and spinal column. Caffeine excites the entire cortex region, the most however the psychic and sensory functions. It removes drowsiness and fatigue, brings on an increase of intellectual activity, perception disposition and a more precise association. This effect is best seen during fatigue, digestion and the depression of activity through alcohol. This fact becomes suggestive through a Graf experience where 10 persons were to accomplish within a fixed time period a certain number of calculations. Diagram 2 shows that after 0.1 g of caffeine which represents about 1 cup of strong coffee, an increase of 4.7% occurred as related to the control, and an increase of 3.1% of solved calculations without error. After 0.2 g of caffeine, this increase is still higher, however, it drops after 0.3 g.

Apart from the psychic sphere, caffeine exerts an effect on the motor centers, it increases the motor activity, which is reflected since various actions become more prompt and more able, for example typists type faster and with fewer mistakes after coffee, but high amounts of coffee can in themselves cause errors in their activity.

Czok and Lang experimented on mice concerning the excitability of the central nervous system by measuring the spontaneous activity on the actograph. After administration of the coffee to the mice at a rate of 10 mg per kg of weight, prepared with 20 g of coffee per 200 ml of water, the spontaneous activity increased during one hour, from 3378 to 6544 movements recorded on the actograph. Many people do not realize this exciting effect, which is still provable by precise functional tests. Through measurement of the excitability of the central nervous system according to Gerlich by means of the electroshock on white rats, one may observe not only the exciting effect of caffeine, but also of chlorogenic acid.

For a certain tension of the alternating current passing through the brain, generalized convulsions occur. The excitability threshold is constant for each animal. The substances changing the excitability of the central nervous system also change the perception threshold of excitement for which the convulsions appear. Thus, caffeine loweres through its stimulating effect on the central nervous system the excitability threshold, which is seen in Diagram 3 through the change of the speed of the curve. Curve 1 represents the excitability threshold for 150 minutes for the controls, curve 2

after 1.25 mg of caffeine per kg of weight, Curve 3 after 2.5 mg per kg and Curve 4 after 3.75 mg per kg of weight. The diagram shows that the curves of the excitability threshold descend unequivocally after administration of the caffeine and according to its dosage.

Diagram 4 represents the effect of chlorogenic acid. Even after the latter, there is observed a lowering of the excitability threshold, of course after greater doses than those of caffeine. The intensity of the effect of the chlorogenic acid represents only 1/6 of the effect after caffeine.

This favorable effect of coffee is used in the intellectuals in the labile nerves, which suffer from a sleep insufficiently deep during the first half of sleep and sleep very deeply in the morning hours, waking up with difficulty in the morning, and their work capability at this moment is very low. Coffee restores the nervous system and the entire organism which allows them to be brought quickly to intense intellectual activity. There are even persons in the afternoon where after lunch there are performed digestions in the gastrointestinal apparatus, a high blood flow in the vessels in the region of the splanchnic nerve, a lowering of the arterial tension and of the blood flow of the central nervous system which results in fatigue and drowsiness. At the present time of the technical revolution and an intensive life, there are few intellectuals who have the possibility of napping in the afternoon. Coffee chases fatigue and drowsiness and allows one to continue to work.

Caffeine produces a stimulating effect also in the respiratory center which is useful fatigue, and on the drowsiness after the waking period preceeded by the application of the hypnotics. A weak sleep is a frequent occurence with intellectuals, just as the application of sedatives of the barbituate series. Hyponotics cause an unfavorable depressing effect for the respiratory center, caffeine removes this unfavorable effect or at least weakens it. Coffee, above all its caffeine component, exerts an effect on the myocard and on the coronary circulation. During the examination of the effect of coffee on the heart, we will be less interested in the puritive effect of caffeine on the sick heart than in the question whether coffee exerts a harmful effect on the healthy heart or not. Through caffeine, coffee acts on the sino-auriculary knot, on the heartbeat and on the ventricular myocard itself. It is true that the stimulus of the sino-auriculary knot would produce a moderate tachycardia, but the simultaneous stimulus of the nuclei of the pneumogastric nerve produces a bradycardia which compensates the two impulses and for this reason, a resasonable amount of coffee does not cause any tachycardia. Tachycardia occurs only during frequent or exaggerated coffee intake. In addition to the influence on the sino auriculary knot, coffee improves the conductability of the heartbeat, strengthens through its positive inotrope effect, the cardiac contractions, improves in this way the activity of the heart and increases the flow per minute. This influence on the heart is

direct. If we add caffeine to the perfusion liquid of the isolated mammal heart, stronger contractions occur and an increase of the systolic flow of the heart.

In the experiment of Ammon and Estler there is produced the increase in the activity of the heart of rats after the administration of 40 mg of caffeine per kg of weight calculated according to the formula of Bretschneider: average arterial tension multiplied by the square root of frequency. For twenty minutes, an evident increase is shown in the activity of the heart. The effect of the coffee on the dilatation of the coronary arteries in man is still discussed. This effect would be important in modern times on infections of the coronary arteries of the heart in intellectuals. Statistics show that in civilized countries we already notice changes in the coronary arteries in youth, often having no clinical symptoms. The flow of the coronary arteries in animals measured directly by the volume of blood or of perfusion liquid flowed, shows that caffeine dilates the coronary arteries and thus improves the coronary circulation.

In the experiments of Sapozkov, carried out on dogs, we have seen the increase, after 10 mg of caffeine per kg of weight, of the normal blood flow up to 45.2 percent without any increase of the peripheric arterial tension. To this effect belongs caffeine through its influence on the smooth muscle of the coronary arteries just as perhaps through its effect carried out on the colaterial circulation of the heart. Recently, it has been shown through experimentation that there is produced, after caffeine, an increase in the rate of non-esterified fatty acids and sometimes also of cholesterol in the blood. By contrast, the rate of esterified fatty acids and triglycerides underwent no change.

Other works also dispute the influence on the rate of cholesterol. The works of Yudkin have showed that the increase of the lipidic fractions in the blood is often brought about more by the absorption of the saccharose in the coffee than by the influence of caffeine. The examination of the activity of the pyruvic kinase in the liver as an index of the lipogenesis after caffeine showed no change of activity, after the saccharose its activity is doubled. This problem is important from the point of view of aterogenesis, but it is still not clear and for this reason we must not wait for the results of later research. Beyond this, the overwhelming part of research had taken place after the administration of caffeine, which does not faithfully reflect in general the effect of coffee. According to certain works, caffeine increases the rate of glucose in the blood and lowers the tolerance in carbohydrates.

Müller-Wieland and Bellet have recently proved that coffee causes no increase in the glycemia in healthy adults or does it only minimally and does not bother the tolerance in carbohydrates.

At present, full of stress, a high number of intellectuals suffer from hypertension. For this reason we must be concerned with the problem whether a person suffering from hypertension can

drink coffee. Consequently, the question is raised as to how coffee acts, or certain of its components, on the arterial system. Opinions on coffee in a patient suffering from hypertension are divergent. Certain clinicians consider coffee as contre-indicated because of its stimulating influence on the central nervous system. For approximately of 30% of the patients, there is produced in the advanced stage of the illness a moderate increase in the arterial tension from 10 to 15 mm Hg, approximately. However, for the greater part of the patients a dose of 1 cup of coffee does not change the arterial tension or it causes a moderate decrease of the systolic and diasystolic arterial tension and of the pulse. The maximal effect occurs during the 40 to 50 minutes and the return to original values after about 2 hours. This effect is explained by the influence of the caffeine contained in the coffee, which dilates the peripheral vessels mainly of the members, kidneys, and cutaneous region. The effect of caffeine is explained by the influence exerted directly on the smooth muscles of the peripheric vessels. In light of these effects, we cannot consider the contre-indication of coffee during hypertension as correct in principle.

It is interesting to note the effect of caffeine exerted on the normal blood flow of the cerebral arteries. Experience has shown that a cup of coffee exerts a favorable effect during certain headaches. During the examination of this phenomenon, we have arrived at the conclusion that the caffeine contained in coffee causes a moderate constriction of the cerebral arteries and thus lowers the blood flow of the brain. This effect of coffee is well used during the suppression of migraine headaches.

As is known from Svec, the migraine headache occurs in three phases. In the first phase, vasoconstricting phase, the cerebral arteries are contracted, which causes the appearance of sight problems, photophobia, scotoma and spots before the eyes. The second phase is shown by a dilation of the arteries, especially in the region of the outer carotid artery. The stimulus of the sensitive receptors in the arteries causes migraine headaches. At the same time, nausea, vomiting, paresthesia, etc. can occur. In the third phase - edematous phase - the headache persists and ceases only after several hours. Caffeine reduces, together with ergotaminic tartrate, through its vasoconstricting effect in the second phase, the amplitude of the pulsating wave and thus moderates the headache. In the lighter cases of the migraine, sometimes one single cup of coffee is sufficient. In pharmacology, the sedative effect of caffeine administered with analgesics is well known.

The effect of coffee exerted on the muscles of the skeleton of the intellectuals is appreciable, because it moderates muscular fatigue. The caffeine contained in coffee increases muscular activity through a direct effect exerted on the striated muscle. During an electric stimulus of the skeletal muscle of the laboratory animals in constant intervals, the strength of the contractions increases through the influence of caffeine. Ergographic experiments performed on man and on animals prove an increase of muscular contractions, an increase in the work ability and a recession of muscular fatigue.

Diagram 6 shows the increase in the work of the isolted heart muscle of the dog after administration of caffeine in a dose of 5 cm.

The consumption of oxygen in the muscle system and the release of thermal energy increase. The experimental works of Ritchie prove that caffeine influences muscular contraction by easily penetrating the muscular cells, by reducing the fixation of calcium on the membranes and by causing the release of calcium from the sercoplasmic reticulum in the tropomyosin.

The result of this is an increase of thermoplastic activity, which increases the contractility of the muscle. This favorable effect of coffee is useful during the before noon part of the activity when the workability begins to diminish rapidly after about 4 hours, as Goldmark and Hopkins have proved and as we see it in Diagram 7.

Coffee at this time can chase muscular fatigue and increase activity. Beside the effect produced directly on the muscle system, the effect of coffee works also on the central nervous system, especially on the cortex.

The effect of coffee, or of its constituents, especially caffeine is explainable through the direct influence exerted on the cells of the organ (brain, cells of the muscle system of the vessels, of the muscle system of the skeleton, partially also of the heart), but in addition to this, also through the effect of the catechol amines released from the coffee. Recently it has been shown that there is produced after administration of the caffeine a release of the endogenous catechol amines which can be proved by their direct measurements in urine and through identification of their decomposition products in urine, as for example vanillyl-mandelic acid. Diagram 8 shows the excretion of vanillyl-mandelic acid in urine in microgram/h according to the experiment of Krieg et al. In 20 healthy persons before and after coffee containing 230 mg of caffeine. After the coffee, an almost double increase was produced of the excretion of vanillyl-mandelic acid.

The effects of caffeine and of the catechol amines on the heart and on the basic metabolism are very similar. If we block the adrenergic beta receptors, the effect of caffeine on the heart and on the basic metabolism diminishes. This proves the effect of caffeine, not only direct, but also, for example, on the heart and on the basic metabolism, through the catechol amines. Men suffering from hyperfunction of the sympathetic nerve react badly for this reason to coffee, the most often by palpatations and by uneasiness.

Another new opinion on the effect of caffeine comes from the idea that the cyclic 3'-5'-adenosinomonophosphate (AMP) is a mediator for the metabolic effect of the catechol amines. The cyclic 3'-5'-AMP is normally found in the tissues where it activates phosphorylase and inhibits glycogenic synthetase by thus favoring glycogenolyse, by then activating the lipase of the fat tissue, accelerates the doubling of the triglycerides and produces free fatty

acids necessary for example in cardiac contraction. The effect of the catechol amines has as a consequence the activation of the cyclase enzyme which leads to the increased formation of 3'-5'-AMP from ATP. The phosphodiesterase enzyme degrades the cyclic 3'-5'-AMP into linear 5'-AMP. Caffeine inhibits this enzyme and also contributes to an increased accumulation of the cyclic 3'-5'-AMP. The latter represents an important mediator of the metabolic processes in the cells as for example the so-called "second messenger" in the cells.

An interesting effect of coffee is produced on the secretions of gastric juice, bile and pancreatic juice. According to experimental and clinical observations, coffee increases these secretions and assists in digestion. The effect produced here is not only that of caffeine, but also other substances present in coffee, mainly substances formed by roasting. These aromatic substances exert, with the help of the taste and olfactory nerves, an effect on the vegetative nerve by stimulating the digestive and intermediary apparatus from which is produced the activation of the upper parts of the nervous system, of the so-called psychic device. These fats are known in psychology and have become famous through the research of Pavlov on conditioned reflexes during the intake of food.

By evaluating the above, we can ponder the question of when coffee can exert a favorable effect on the organism of the intellectual. In the morning, in the waking hour, it helps the transition of the organism from the vagotrope phase to the sympathicotrope phoase, causes a stimulation of the nervous system and makes possible a prompt arrival at intellectual work. In the morning, at the time of the lowering of the work intensity, its stimulating effect on the cortex and on the skeletal muscle chases fatigue and increases working ability. In the afternoon, a normal decrease in the blood flow of the brain is normally present, as a result of digestion, which again leads to fatigue and drowsiness. Coffee taken at this time again chases fatigue, and in addition to this it aids digestion through its activity on the secretion of the digestive juices. There is much discussion on the consumption of coffee in the evening hours, and there is little agreement. It is certainly known that certain persons do not tolerate coffee in the evening, because it interfers with their nocturnal sleep, but that others tolerate it very well.

The experiments of the Pavlov school have shown that doses of caffeine not exceeding 5 cm (i.e. a small cup of coffee, exert a depressing influence on the cortex, aid in slumber and sleep and act synergistically with barbituates. For this reason, persons who cannot tolerate the normal dose of coffee in the evening should try to use coffee in reduced dosage.

In conclusion, it can be affirmed that coffee is a most innocent means of toxicomania, it forms no habit, causes no symptoms of a change in habits and does not at all change the personality and the human character. The toxicity of coffee is only very low, even

insignificant. Until now, only two cases of fatal poisoning are known: in the first case through an error, 3.2 g of caffeine had been administered by the; in the other case, with the intention of suicide, 50 g of caffeine had been taken. According to experiments performed on animals, the lethal dose of coffee for man would be 1 kg approximately of roasted coffee, which equals 100 cups at least of strong coffee. In 1938, the German pharmacological society in Berlin was concerned with the possible harmful effect of coffee. At the end of the conference, it was established that normal coffee produces no harmful influence of long or short range on the human organism. After 30 years, these conclusions have been examined and have been shown to be correct with certain exceptions concerning coffee during certain illnesses.

Based on facts, one should consider, in the battle against alcoholism, installing and enlarging the network of coffee houses and tea rooms with the use of coffee in a cultural atmosphere.

Substance	Roasted coffee in g per 100 g	Roasted coffee in g per brew of 100 g
Caffeine	1.35	1.08
Chlorogenic acid	4.1	3.3
Coffee fat	12.5	6.4
Cafeic acid	0.95	0.75
Quinic acid	0.95	0.75
Trigonelline	0.45	0.36
Choline	0.04	0.032
Potassium and others	1.6	1.3

Diagram 1 according to Kühne, 1962

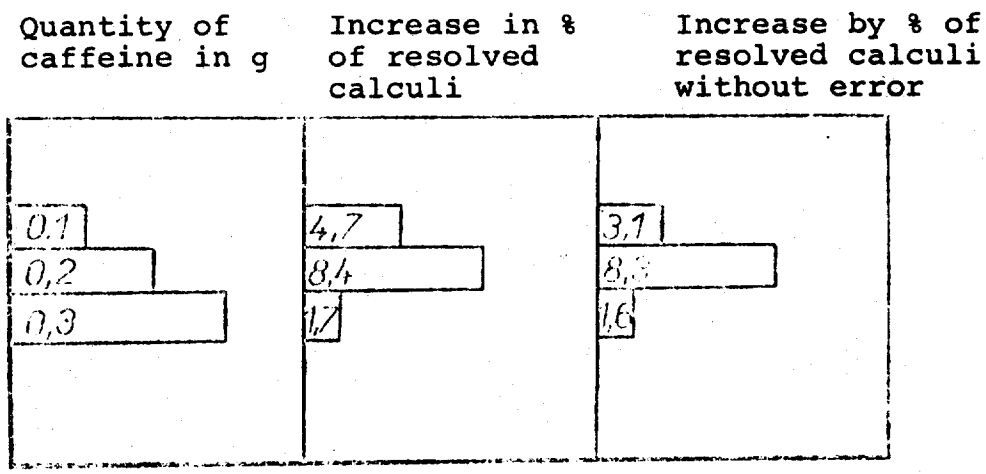

Diagram 2. Average percent of change in the test results of calculation for 3 hours in 10 persons after application of different doses of caffeine (according to Graf, 1961).

Excitability threshold

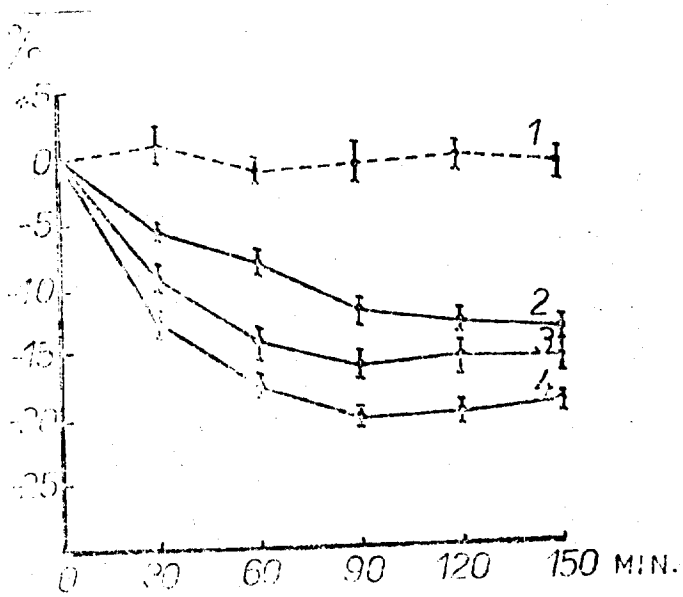

Diagram 3. Change in the excitability threshold by percent in rats after application of caffeine per os. (according to Czok, 1961). Key: 1) controles, 2) caffeine 1.25 mg/kg, 3) caffeine 2.50 mg/kg, 4) caffeine 3.75 mg/kg.

Excitability threshold

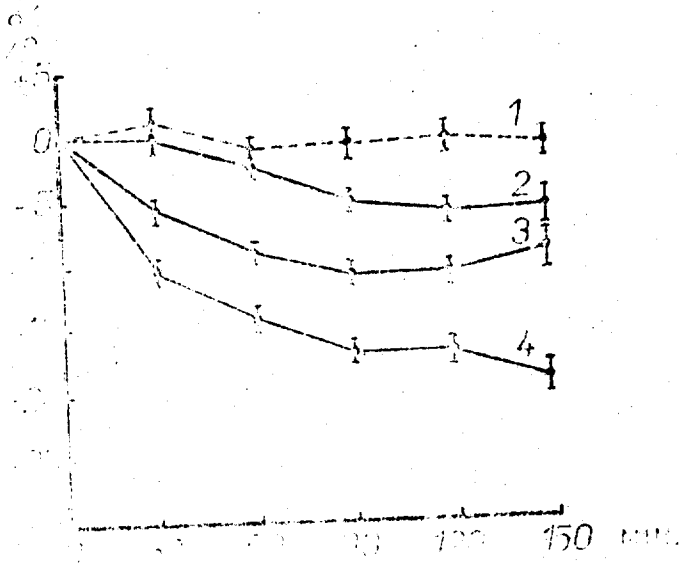

Diagram 4. Change in the excitability threshold in percentage in rats after application of caffeine per os. (according to Czok, 1961). 1) controls, 2) chlorogenic acid 3.75 mg/kg, 3) chlorogenic acid 7.50 mg/kg, 4) chlorogenic acid 15.00 mg/kg.

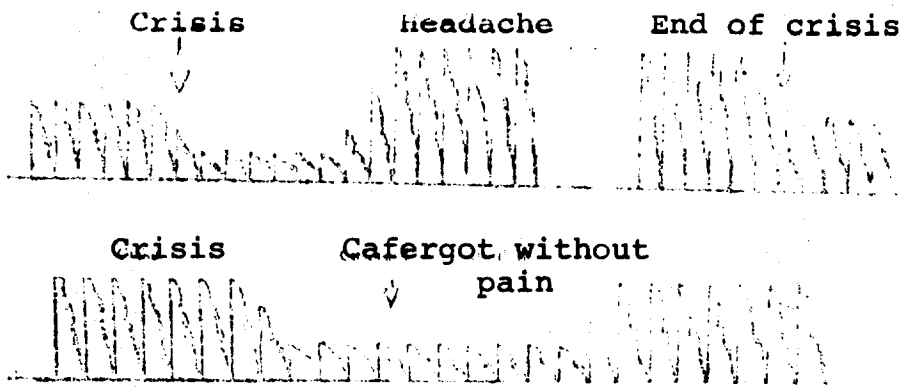

Diagram 5. Influence of the cafergot. 1 mg of tartrateergotamine + 0.1 g of caffeine) on the migrane headache (according to Svec, 1956).

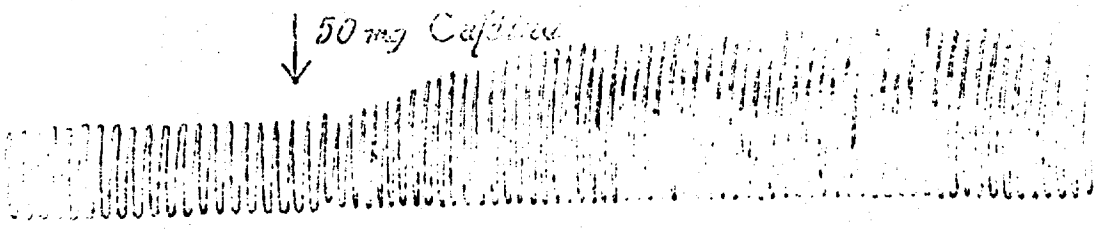

Diagram 6. Influence of caffeine (1:500) on the activity of the isolated myocard of the dog. (according to Mozgov, 1948).

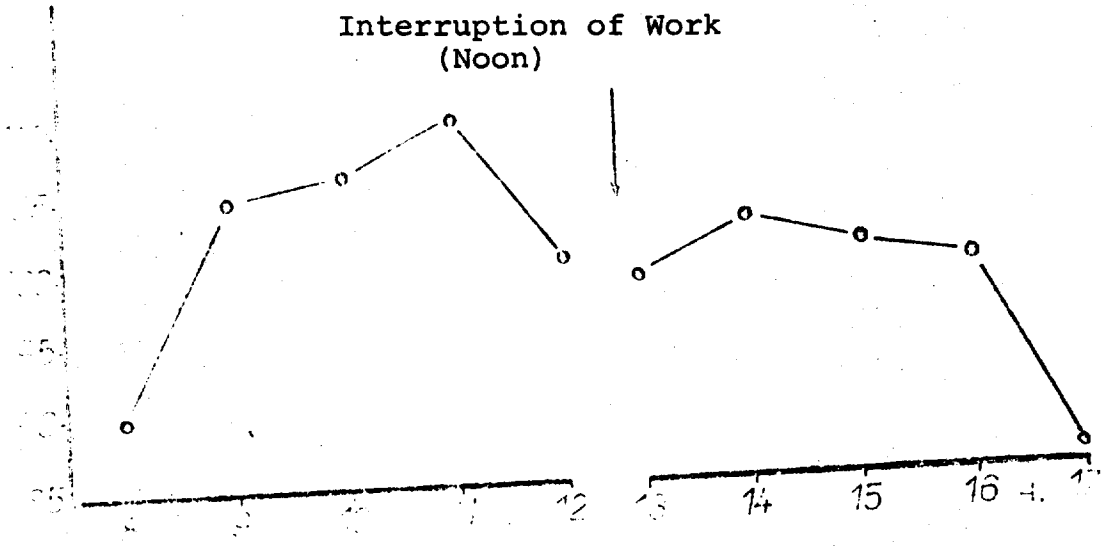

Diagram 7. Effectivity diagram of work in two large factories in percentage of the effectivity per hour (according to Goldmark and Hopkinds, 1961).

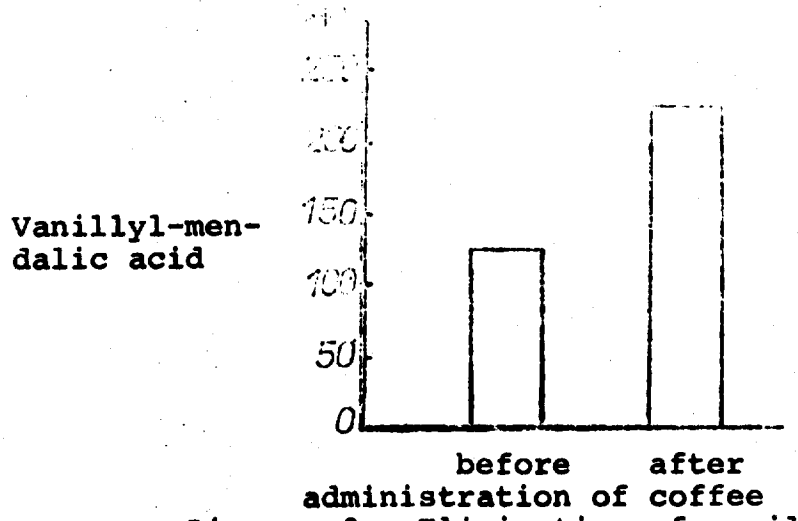

Diagram 8. Elimination of vanillyl-mendalic acid in urine after application of brewed coffee containing about 230 mg of caffeine (N=20) (according to Krieg et al. 1969).

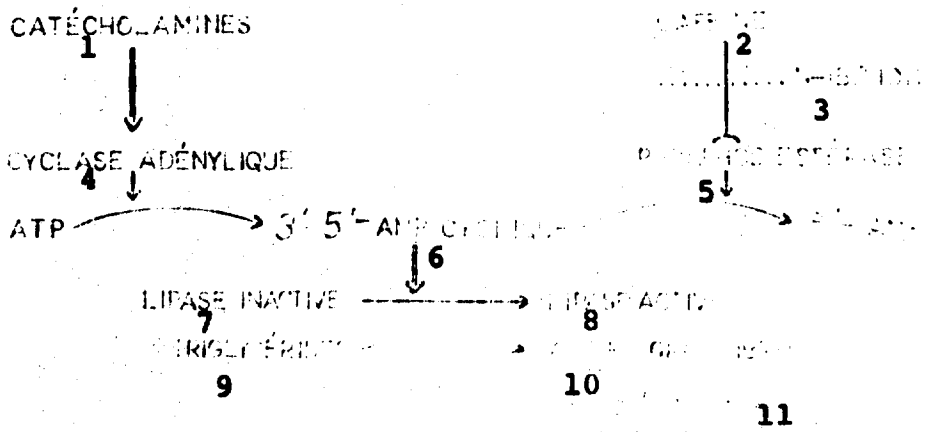

Diagram 9. Key: 1) catecholamines, 2) caffeine, 3) inhibition, 4) cyclase adenylic, 5) phosphodiesterase, 6) AMP cyclic, 7) inactive lipase, 8) active lipase, 9) triglycerides, and 10) free fatty acids, 11) glycerol. Effect of the 3'-5'-AMP and influence of the catecholamines and caffeine (according to Hynie et al., 1966).

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L'importance du café pour l'organisme de l'intellectuel

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A l'époque actuelle, le café est devenu l'un des comestibles le plus fréquemment consommés, bien que l'histoire de sa consommation per l'homme en Europe — en différence de l'alcool et de tabac — ne dépasse que peu 400 ans. Dans les recherches de l'influence du café sur l'organisme humain, un tournant s'est produit en 1820 quand Runge a découvert dans le café la caféine comme le principe, et, en 1899, Fischer à défini sa formule structurale. Dès là, des discussions sont en cours au sujet de l'utilité ou de la nocivité du café pour l'organisme humain et pas un jour ne passe que le médecin ne devrait pas répondre aux questions des malades s'il leur est permis, dans leur maladie, de prendre du café ou non. Ces problemes sont vraiment importants, parce qu'un médecin non-initié aux effets du café et interdisant d'une maniere globale le cafe-boisson causera plus de dommage que de profit. Il est bien sur que le cafe possède maintes bonnes qualites, qu'il produit un effet favorable sur l'organisme humain, mais qu'il peut être nuisible, parfois, en suite d'une fausse application, pour cette raison on doit connaitre exactement tous ses effets produits sur l'organisme.

Dans notre rapport, nous aurons pour l'objectif les effets que le café produit sur l'organisme des intellectuels. C'est la couche sociale qui exploite le plus, chaque jour peut-etre, les effets bénéfiques du café. On peut caractériser l'intellectuel comme un homme exercant l'activité intellectuelle la plus compliquée avec les éléments prédominants de l'activité créatrice. Cette activite créatrice exige au point de vue psychologique, un haut degré de perception, concentration, abstraction et de disposition associante. Il est alors naturel qu'un intellectuel cherche des moyens à intensifier les capacités mentionnées ci-dessus ou bien à les renouveller, s'il est fatigué en suite de l'activité intellectuelle épuisante et ses capacités psychiques s'abaissent.

Une expérience de longues années a démontré que c'est le café qui possède ces qualités exigées.

On croyait dès la découverte de la caféine dans le café que le principe actif, voire même unique, dans le café soit justement la caféine. Aujourd'hui, on sait que le café est un comestible complexe contenant à côté de la caféine encore des substances diverses. La caféine ne se trouve pas dans le café en une forme libre, au contraire fixée ensemble avec le potassium et l'acide chlorogénique en forme d'une substance complexe. Outre ces composantes, le café contient encore l'acide caféique, l'acide quinique, la trigonelline, la graisse du café et la choline et beaucoup d'autres comme il se fait voir au diagramme 1.

Substance	Café torrefié	
	En g par 100 g	En g par infusion de 100 g
Caféine	1,35	1,08
Acide chlorogénique	4,1	3,3
Graisse du café	12,5	6,4
Acide caféique	0,95	0,75
Acide quinique	0,95	0,75
Trigonelline	0,45	0,36
Choline	0,04	0,032
Potassium et d'autres	1,6	1,3

Diagramme 1 (D'après Kühne, 1962).

Le taux de la caféine dans le café torréfié fait de 1,3 à 1,4 g pour-cent, dans l'infusion de 100 g de café 1,08 g pour-cent, le taux de l'acide chlorogénique fait 4,1 g %, et 3,3 g % dans l'infusion. En suite de la fixation de la caféine, il se produit son plus lent dégagement et sa plus lente résorption dans l'intestin; de ce fait, l'effet du café est plus lent et moins intensif que celui de la caféine pure [1].

L'effet peut-être le plus important chez les intellectuels et le plus apprécié est l'effet exercé sur le système nerveux central. Les substances contenues dans le café — et cela non seulement la caféine, comme on verra ci-dessous — exercent un effet stimulant sur l'écorce cérébrale, sur les centres végétatifs thalamiques, dans la moelle allongée et épinière. La caféine excite toute la région de l'écorce cérébrale, le plus cependant les fonctions psychiques et sensorielles. Elle chasse le somnolence et la fatigue, entraine une augmentation de l'activité intellectuelle, de la disposition perceptive et une association plus précise [2]. Cet effet est à constater le mieux pendant la fatigue, la digestion et la dépression de l'activité par l'alcool [3]. Ce fait devient suggestif par une expérience de Graf où 10 personnes devaient accomplir dans un délai fixé un certain nombre de calculs. Le diagramme 2 démontre qu'après 0,1 g de caféine ce qui représente une tasse à peu près de café fort, une augmentation de 4,7 % de calculs résolus est survenue par rapport au contrôle, et une augmentation de 3,1 % le calculs résolus sans faute. Après 0,2 g de caféine, cette augmentation est plus élevée encore, tout de même, elle s'abaisse déja après 0,3 g [4].

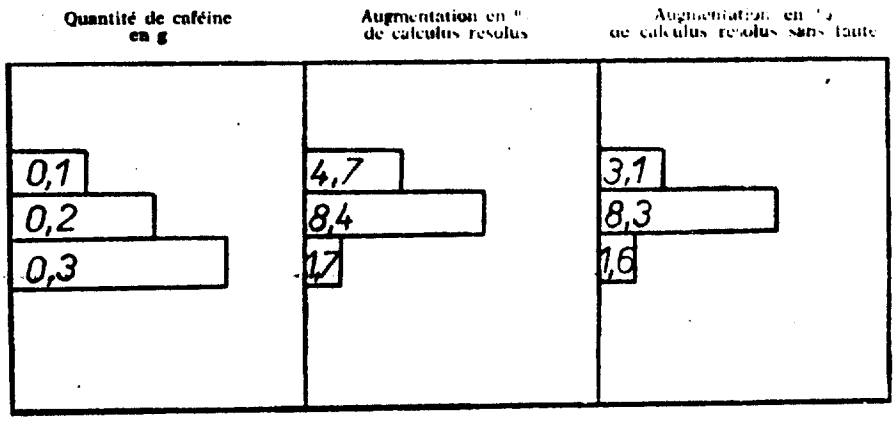

Diagramme 2. — % moyen de changement dans les résultats d'essai de calcul durant 3 h chez 10 personnes après application de différentes doses de caféine. (D'après Graf, 1961).

tral à l'aide de la mesure de l'activité spontanée à l'actographe. Après l'administration du café aux souris en un taux de 10 mg d'infusion par kg de poids, préparée de 20 g de café par 200 ml d'eau, l'activite spontanée augmentait, au cours d'une heure, de 3378 à 6544 mouvements enregistrés à l'actographe. Bien de personnes ne se rendent pas compte de cet effet excitatif, prouvable tout de même par des essais fonctionnels précis. Par mesure de l'excitabilité du système nerveux central d'après Gerlich à l'aide de l'électro-choc sur les rats blancs, on peut observer non seulement l'effet excitatif de la caféine, mais aussi de l'acide chlorogénique [5].

Pour une certaine tension du courant alternatif passant par le cerveau surviennent des convulsions généralisées. Le seuil de la excitabilité est pour chaque animal constant. Les substances changeant l'excitabilite du

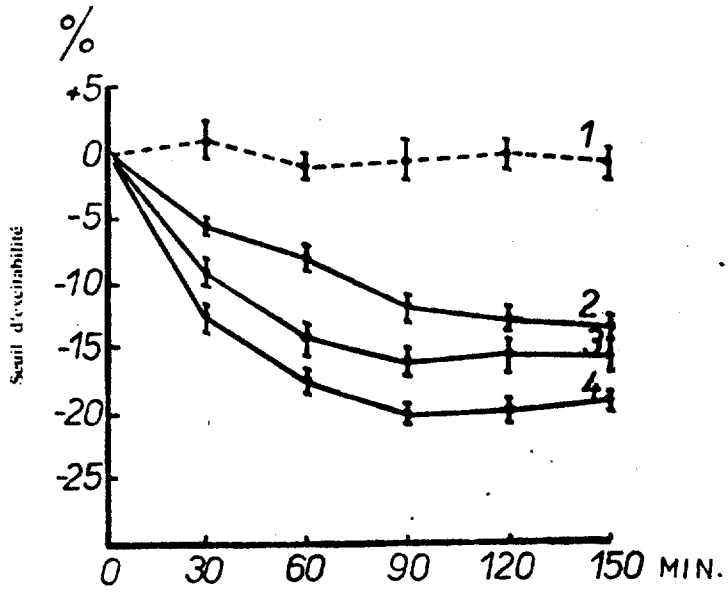

Diagramme 3. — Changement du seuil d'excitabilité en % chez les rats après application de la caféine per os. (D'après Czok, 1961). - 1) Contrôles. 2) Caféine 1,25 mg/kg. 3) Caféine 2,50 mg/kg. 4) Caféine 3,75 mg/kg.

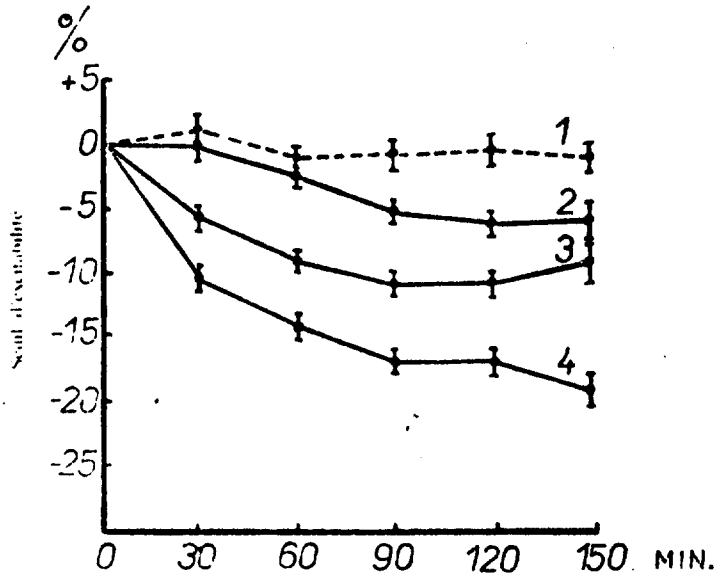

Diagramme 4. — Changement du seuil d'excitabilité en % chez les rats après application de la caféine per os. (D'après Czok, 1961). - 1) Contrôles. 2) Ac. chlorogénique 3,75 mg/kg. 3) Ac. chlorogénique 7,50 mg/kg. 4) Ac. chlorogénique 15,00 mg/kg.

système nerveux central changent aussi le seuil de perception de l'excitation pour laquelle apparaissent les convulsions. Ainsi, la caféine abaisse par son effet excitatif sur le système nerveux central le seuil de l'excitabilité ce qui se fait voir au diagramme 3 par le changement de l'allure de la courbe. La courbe 1 représente le seuil de l'excitabilité au cours de 150 minutes pour les contrôles, la courbe 2 après 1,25 mg de

caféine par kg de poids, la courbe 3 après 2,5 mg par kg et la courbe 4 après 3,75 mg par kg de poids. Le diagramme démontre que les courbes du seuil d'excitabilité descendent univoquement après l'administration de la caféine et en dépendance de son dosage.

Le diagramme 4 représente l'effet de l'acide chlorogénique. Même après ce dernier, il se fait observer un abaissement du seuil d'excitabilité, bien entendu après des doses supérieures à celles de caféine. L'intensité de l'effet de l'acide chlorogénique représente seulement un sixième de l'effet après la caféine.

Cet effet favorable du café est exploité chez les intellectuels aux nerfs labiles, qui souffrent d'un sommeil insuffisamment profond dans la première moitié du sommeil et dorment très profondement dans les heures matinales, ne s'éveillent que difficilement le matin, et leur capacité de travail en ce moment est très basse. Le café-boisson ranime le système nerveux et l'organisme entier ce qui leur permet de s'entraîner vite à l'intensive activité intellectuelle. Il y en a de même aussi l'après-midi où se produit, le repas de midi absorbé, la digestion dans l'appareil gastro-intestinal, un flux sanguin élevé dans les vaisseaux de la région du nerf splanchnique, un abaissement de la tension artérielle et du flux sanguin du système nerveux central ce qui résulte en fatigue et somnolence. A l'époque actuelle de la révolution technique et d'une vie intensive, il y a peu d'intellectuels qui ont la possibilité de dormir après-midi. Le café-boisson chasse la fatigue et somnolence et permet de continuer au travail.

La caféine produit un effet excitatif aussi sur le centre respiratoire ce qui est salutaire pendant la fatigue, et sur la somnolence après le reveil précédé de l'application des hypnotiques. Un faible sommeil est un phénomène fréquent chez les intellectuels, de même que l'application des sédatifs de la série barbiturique. Les hypnotiques évoquent un effet dépressif défavorable pour le centre respiratoire, la caféine supprime cet effet défavorable ou l'affaiblit au moins[9-10]. Le café, surtout sa composante caféinique, exerce un effet sur le myocarde et sur la circulation coronarienne. Pendant l'examen de l'effet du café sur le coeur, nous nous intéressons moins à l'effet curatif de la caféine sur le coeur malade qu'à la question si le café-boisson exerce ou non un effet nuisible sur le coeur sain. Par la caféine, le café agit sur le noeud sino-auriculaire, sur le système de commande du coeur et sur le myocarde ventriculaire lui-même. Il est vrai que l'excitation du noeud sino-auriculaire produirait une tachycardie modérée, mais l'excitation simultanée des noyaux du nerf pneumogastrique produit une bradycardie ce qui compense les deux impulsions et, pour cette raison, un café-boisson raisonnable ne cause aucune tachycardie[11]. La tachycardie ne survient que pendant un café-boisson fréquent ou exagéré. Outre l'influence sur le noeud sino-auriculaire, le café améliore la conductibilité du système de commande du coeur, renforce, par son effet inotrope positif, les contractions cardiaques, améliore de cette façon l'activité du coeur et augmente le débit par minute. Cette influence sur le coeur est directe. Si l'on ajoute de la caféine au liquide de perfusion du coeur isolé de mammifère, des contractions plus puissantes se présentent et une augmentation du débit systolique du coeur.

Dans l'expérience d'Ammon et d'Estler[12] se produit l'augmentation de l'activité du coeur des rats après l'administration de 40 mg de caféine par kilogramme de poids calculé d'après la formule de Bretschneider: tension artérielle moyenne multipliée par la racine carrée de la fréquence. Au cours de 70 minutes, une augmentation évidente s'est produite de l'activité du coeur. L'effet du café sur la dilatation des artères coronaires chez l'homme est encore discuté. Cet effet aurait d'importance à l'époque contemporaine des affections des artères coronaires du coeur chez les intellectuels. Les statistiques démontrent que dans les pays civilisés on constate des changements des artères coronaires déjà chez la jeunesse, n'évoquant souvent aucuns symptômes cliniques. Le débit des artères coronaires chez les animaux mesuré directement par le volume de sang ou de liquide de perfusion passés montre que la caféine dilate les artères coronaires et améliore ainsi la circulation coronarienne.

Dans les expérience de Sapozkov[13], faites sur les chiens, on a vu augmenter, après 10 mg de caféine par kg de poids, le flux sanguin normal jusque de 45,2 % sans aucune augmentation de la tension artérielle périphérique. A cet effet prend part la caféine par son influence sur le muscle lisse des artères coronaires de même que

peut-être par son effet exerce sur la circulation colatérale du coeur. Récemment, il s'est montré pendant l'expérimentation qu'il se produit, après la caféine, une augmentation du taux des acides gras non-estérifiés et parfois aussi de la cholestérole dans le sang. Par contraire, le taux des acides gras estérifiés et des triglycérides n'a subi aucun changement [14-15-16].

D'autres travaux contestent aussi l'influence sur le taux de la cholestérole [17-18]. Les travaux de Yudkin [19] ont montré que l'augmentation des fractions lipidiques dans le sang est entrainée souvent plutôt par l'absorption du saccharose dans le café que par l'influence de la caféine. L'examen de l'activité de la kinase pyruvique dans le foie comme un indice de la lipogenèse après la caféine n'a montré aucun changement de l'activité, après le saccharose son activité est doublée. Ce problème est important au point de vue de la atérogenèse, mais il n'est pas encore tiré au clair et on doit attendre pour cette raison les résultats des recherches ultérieures. Outre cela, la partie prépondérante de recherches avaient lieu après l'administration de la caféine ce qui ne reflète pas fidèlement en général l'effet du café. D'après certains travaux, la caféine augmente le taux du glucose dans le sang et abaisse la tolérance en glucides [7-8].

Müller-Wieland et Bellet [20] ont prouvé récemment que le café-boisson n'évoque aucune augmentation de la glycémie chez les adultes sains ou ne le fait que minimalement et ne trouble pas la tolérance en glucides.

A l'époque actuelle, pleine de stress, un nombre d'intellectuels élevé souffrent de la hypertension, pour cette raison on doit s'occuper du problème si l'homme souffrant de hypertension peut boire du café. Par conséquent, le question se pose comment agit le café, ou certaines de ses composantes, sur le système artériel. Les avis sur le café-boisson chez un malade souffrant d'hypertension divergent. Certains cliniciens considèrent le café comme contre-indiqués à cause de son influence excitative sur le système nerveux central. Pour une partie de malades qui fait 30 % à peu près, il se produit dans le stade avancé de la maladie une augmentation modérée de la tension artérielle de 10 à 15 mm Hg environ. Cependant, pour la plupart de malades une dose de 1 tasse de café ne change point la tension artérielle ou elle évoque un abaissement modéré de la tension artérielle systolique et diasystolique et du pouls. L'effet maximal se présente au cours de 40 à 50 minutes et la rentrée aux valeurs primitives après 2 heures à peu près. Cet effet est explicable par l'influence de la caféine contenue dans le café, qui dilate les vaisseaux périphériques surtout des membres, des reins et de la région cutanée. On s'explique l'effet de la caféine par l'influence exercée directement sur les muscles lisses des vaisseaux périphériques. Vu ces effets, on ne peut pas considérer la contre-indication du café-boisson pendant la hypertension comme correcte en principe [3].

Intéressant est l'effet de la caféine exercé sur le flux sanguin normal des artères cérébrales. L'expérience a montré qu'une tasse de café exerce un effet favorable pendant certains maux à la tête. Pendant l'examen de ce phénomène on en est arrivé à la conclusion que la caféine contenue dans le café provoque une constriction modérée des artères cérébrales et abaisse ainsi le flux sanguin du cerveau. Cet effet du café est bien utilisable pendant la suppression des crises migraineuses.

Comme on sait, d'après Svec [2] la crise migraineuse se déroule en 3 phases, dans la première phase — phase vasoconstrictrice — les artères cérébrales sont rétrécies ce qui cause l'apparision des troubles de la vision, photophobie, scotome et les points scintillants devant les yeux. La deuxième phase se manifeste par une dilatation des artères, surtout dans la région de l'artère carotide extérieure. L'excitation des récepteurs sensitifs dans les artères évoque de maux de tête migraineux. Parallèlement peut se produire la nausée, le vomissement, les paresthésies, etc. Dans la troisième phase — phase oedémateuse — le mal à la tête persiste et ne cesse qu'après quelques heures. La caféine diminue, ensemble avec le tartrate ergotaminique, par son effet vasoconstricteur dans la deuxième phase l'amplitude de l'onde pulsatile et modère ainsi le mal à la tête. Dans les cas plus légers, de la migriane, il suffit quelque fois une seule tasse de café. On connait bien en pharmacologie l'effet sédatif de la caféine administrée ensemble avec les analgétiques [21].

L'effet du café exercé sur les muscles du squelette des intellectuels est appréciable, parce qu'il modère la fatigue musculaire [22]. La caféine contenue dans le café augmente l'activité musculaire par un effet direct

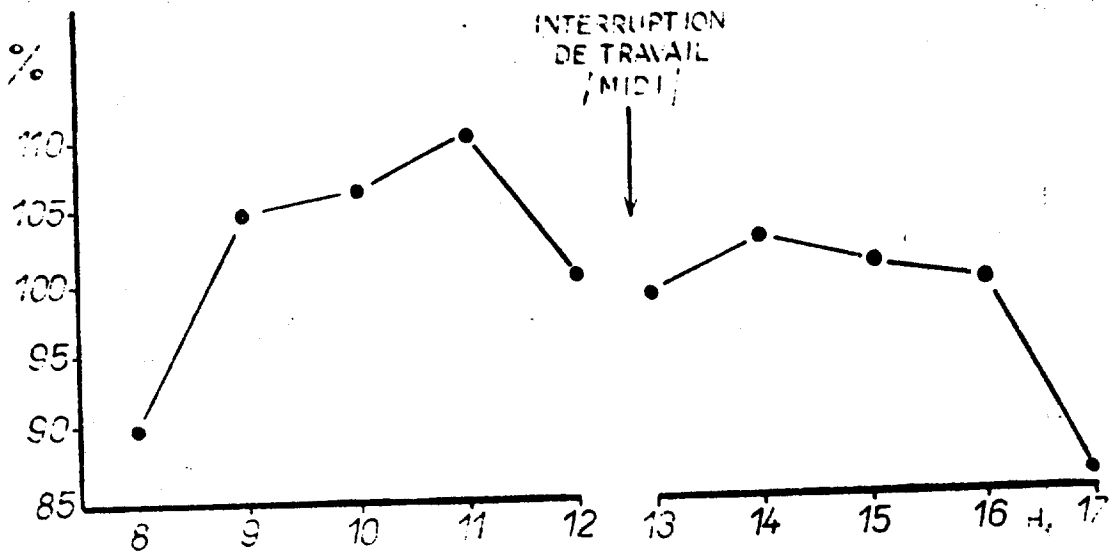

Diagramme 7. — Courbe de l'effectivité du travail dans 2 grandes usines en % de l'effectivité par heure. (D'après Goldmark et Hopkins, 1961).

l'administration de la caféine un dégagement des catécholamines endogènes ce qu'on peut prouver par leur mesure directe dans l'urine ou par identification de leurs produits de décomposition dans l'urine, comme par exemple de l'acide vanillyl-mandélique. Le diagramme 8 représente l'excrétion de l'acide vanillyl-mandélique dans l'urine en g/h suivant l'expérience de Krieg et Coll.[illegible] chez 20 personnes saines avant et après le café-boisson contenant 230 mg de caféine. Après le café une augmentation presque double s'est produite de l'excrétion de l'acide vanillyl-mandélique.

Les effets de la caféine et des catécholamines sur le coeur et sur le métabolisme de base sont très similaires. Si l'on bloque les beta-récepteurs adrénergiques[illegible] l'effet de la caféine sur le coeur et sur le métabolisme de base diminue. Cela prouve l'effet de la caféine non seulement direct, mais aussi [illegible] par exemple sur le coeur et sur le métabolisme de base, par les catécholamines. Les [illegible] de l'hyperfonction [illegible] réagissent pour [illegible] au café-boisson, le plus [illegible] et par une [illegible].

Un [illegible] sur l'effet de la caféine [illegible] que le 3'-5'-adénosinomonophosphate (AMP) cyclique est un [illegible] métaboliques des catécholamines. Le 3'-5'-AMP cyclique se trouve normalement dans les tissus où il active la phosphorylase et inhibe la synthétase glycogénique en favorisant ainsi la glycogénolyse, en activant ensuite la lipase du tissu de graisse, accélère le dédoublement des triglycérides et produit des acides gras libres nécessaires par exemple à la contraction cardiaque. L'effet des catécholamines a pour conséquence l'activation de l'enzyme cyclase ce qui mène à la formation augmentée du 3'-5'-AMP à partit de l'ATP. L'enzyme phosphodiestérase dégrade le 3'-5'-AMP cyclique en 5'-AMP linéaire. La caféine inhibe cet enzyme et contribue aussi à une accumulation élevée du 3'-5'-AMP cyclique. Ce dernier représente un important médiateur des procédés métaboliques dans les cellules comme par exemple le soi-disant « second messenger » dans les cellules[illegible].

Un effet intéressant du café se produit sur la sécrétion du suc gastrique, de la bile et du suc pancréatique[illegible]. D'après les observations expérimentales et cliniques, le café augmente ces sécrétions et favorise la digestion. L'effet produit ici est non-seulement celui de la caféine, mais aussi d'autres substances présentes dans le café, surtout des substances formées par la torréfaction. Ces substances aromatiques exercent, à l'aide des nerfs gustatifs et olfactifs, un effet sur les nerfs végétatifs en stimulant l'appareil

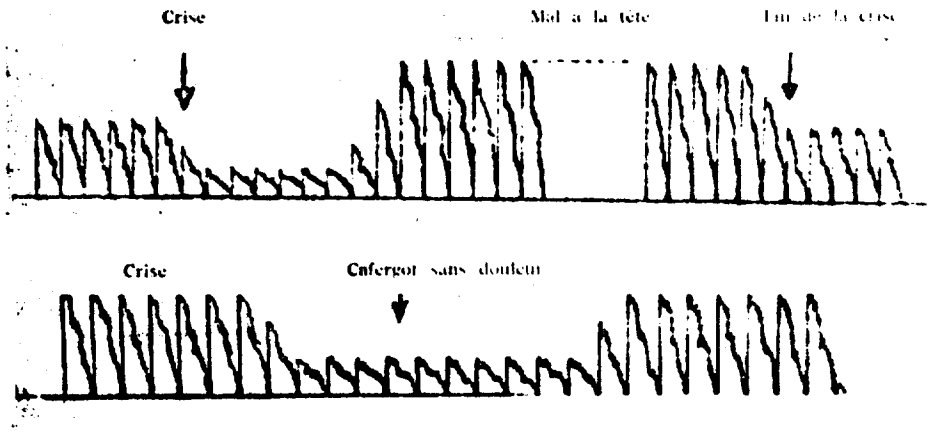

Diagramme 5. — Influence du cafergot (1 mg de tartrate ergotaminique + 0,1 g de caféine) sur la crise de migraine. (D'après Svec, 1956).

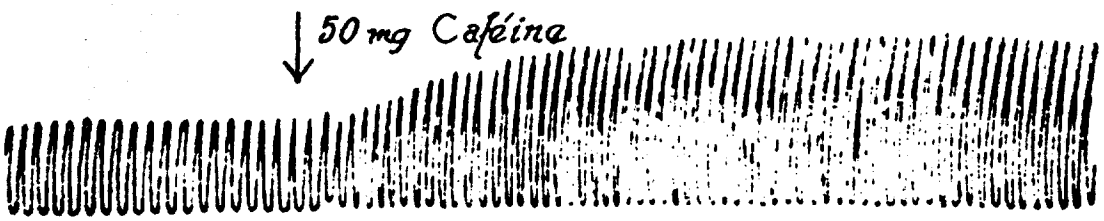

Diagramme 6. — Influence de la caféine (1:500) sur l'activité du myocarde isolé du chien. (D'après Mozgov, 1948).

exercé sur le muscle strié. Pendant une excitation électrique du muscle du squelette des animaux de laboratoire dans des intervalles constants, la puissance des contractions augmente par l'influence de la caféine. Les expériences ergographiques faites sur les hommes et sur les animaux prouvent un agrandissement des contractions musculaires, une augmentation de la capacité de travail et un recul de la fatigue musculaire. Le diagramme 6 fait voir l'augmentation du travail du muscle du coeur isolé du chien après l'administration de la caféine en une dose de 5 centigrammes [23].

La consommation de l'oxygène dans la musculature et le dégagement de l'énergie thermique augmentent. Les travaux expérimentaux de Ritchie [5] prouvent que la caféine influence la contraction musculaire en pénétrant facilement dans les cellules musculaires, en réduisant la fixation du calcium sur les membranes et en effectuant le dégagement du calcium à partir du réticulum sarcoplasmique en la troponiyosine [24].

Le résultat en est une augmentation de l'activité thermoplastique ce qui augmente la contractilité du muscle. Cet effet favorable du café est bien utilisable pendant la partie d'avant-midi de l'activité ou la capacité de travail commence à se diminuer rapidement après 4 heures à peu près, comme l'ont prouvé Goldmark et Hopkins et comme on le voit au diagramme 7 [].

Le café-boisson à cette époque peut chasser la fatigue musculaire et augmenter l'activité. A côté de l'effet produit directement sur la musculature, l'effet du café s'exerce aussi sur le système nerveux central, surtout sur l'écorce cérébrale [25].

L'effet du café, ou de ses pricipes, surtout de la caféine est explicable par l'influence directe exercée sur les cellules de l'organe (le cerveau, les cellules de la musculature des vaisseaux, de la musculature du squelette, partiellement aussi du coeur), mais outre cela aussi par l'effet des catecholamines dégagées le café-boisson [].

Récemment, il s'est montré qu'il se produit après

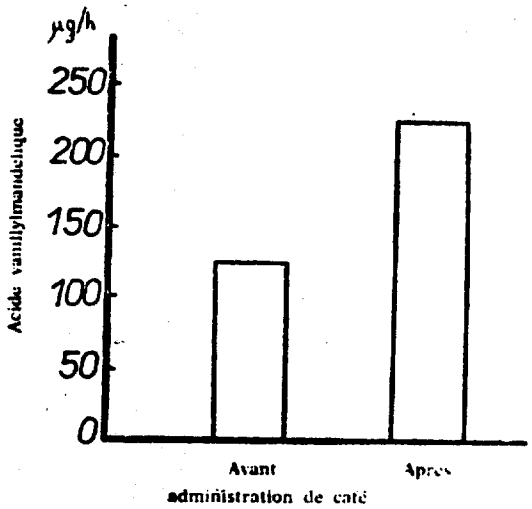

Diagramme 8. — Elimination de l'acide vanillylmandélique dans l'urine après application de l'infusion du café contenant environ 230 mg de caféine (N = 20). (D'après Krieg et Al., 1969).

digestif et intermédiaire d'où se produit l'activation des parties supérieures du système nerveux — du soidisant — appareil psychique. Ces faits sont connus dans la psychologie et sont devenus renommés par les recherches de Pavlov sur les réflexes conditionnés pendant l'absorption de la nourriture.

En évaluant les sus-dit, on peur réfléchir sur la question quand le café peut-il exercer un effet favorable sur l'organisme de l'intellectuel. Le matin, dans les heures de réveil, il favorise la transition de l'organisme de la phase vagotrope à la phase sympathicotrope, évoque une stimulation du système nerveux et rend possible un prompte entraînement au travail intellectuel. Dans la matinée, au temps de l'abaissement de l'intensité de travail, son effet excitatif sur l'écorce cérébrale et sur le muscle de squelette chasse la fatigue et augmente la capacité de travail. Après-midi, un recul du flux sanguin normal du cerveau se présente habituellement en suite de la digestion, ce qui mène de nouveau à la fatigue et somnolence. Le café-boisson pris en ce temps chasse de nouveau la fatigue, outre cela il favorise la digestion par son activité sur la sécrétion des sucs digestifs. On discute beaucoup la consommation du café dans les heures de soir et il n'y en est pas d'unanimité. Il est bien connu que certains hommes ne supportent pas le café le soir, parce qu'il dérange leur sommeil nocturne, les autres, le supportent très bien.

Les expériences de l'école de I. P. Pavlov ont montré que les doses de caféine ne dépassant 5 centigrammes, c.à.d. une petite tasse de café, exercent une influence depressive sur l'écorce cerebrale, favorisent l'assoupissement et le sommeil et agissent synergiquement avec les barbituriques. Pour cette raison, les personnes ne supportant pas la dose normale de café le soir, devraient essayer de prendre du café en dose réduite.

En concluant, on peut affirmer que le café est un moyen de toxicomanie le plus innocent - il ne constitue aucune habitude ni

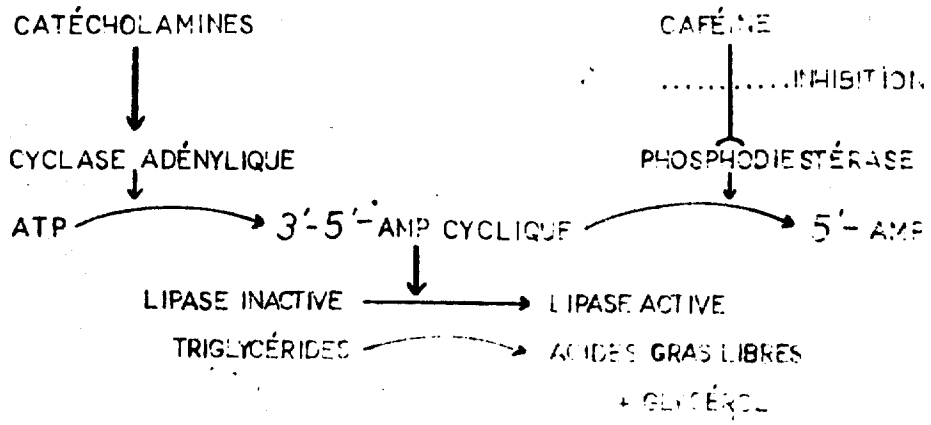

Diagramme 9. — Effet du 3'-5'-Amp et influence des catecholamines et de la caféine. (D'après Hynie et Coll., 1966).

ne possède des symptomes de déshabitude et ne change point la personnalité et le caractère humain ». La toxicité du café n'est que très basse, jusque insignifiante. Jusqu'à présent, on ne connait que deux cas de l'empoisonnement mortel; dans le premier cas, on avait administré, par erreur, 3,2 g de caféine i.v., dans l'autre, on a pris, dans l'intention de se suicider, 50 g de caféine. Suivant les expériences faites sur les animaux, la dose letale de café pout l'homme ferait 1 kg à peu près de café torréfié ce qui répond à 100 tasses minimum de café fort ». En 1938, la Société pharmacologique allemande à Berlin s'occupait de l'influence nuisible eventuelle du café-boisson. En conclusion du congrès, on constatait que le café-boisson normal ne produit aucune influence nuisible de courte ou de longue durée sur l'organisme humain ». Après 30 ans, ces conclusions on été soumises au contrôle et se sont montrées correctes à certaines exceptions près du café-boisson pendant certaines maladies [illegible].

Se basant sur l'exposé, on devrait considerer, dans la lutte anti-alcoolique, d'installer et d'élargir le réseau de caféteries et de salles de thé avec la consommation de café dans un milieu culturel.

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INTERVENTO SULLA RELAZIONE DEL PROF. BUCKO

Prof. SCARSELLI.

In quest'ultima relazione abbiamo sentito parlare di un'azione coronarodilatatrice del caffè e della caffeina. Poichè io sono geriatra ma mi occupo soprattutto di malattie cardiovascolari, penso che sia doveroso da parte mia far rilevare che questa azione benefica della caffeina sulla circolazione coronarica non è nota in quanto, a prescindere dall'azione vasculotropa diretta, esiste indubbiamente una azione mediata dovuta alla liberazione di catecolamine e di NEFA. Questa azione nel coronaropatico è pericolosa per l'insorgenza di aritmie gravi che può scatenare, come ho accennato nella mia relazione.

Ho sentito parlare di acidi grassi liberi che dopo assunzione di caffè sarebbero dovuti più all'azione del saccarosio che a quella della caffeina. In effetti è noto che la caffeina determina la liberazione di acidi grassi liberi per azione diretta e per azione mediata dalle catecolamine di cui stimola l'aumento, mentre la somministrazione di zucchero con l'apporto di radicali glicerofosforici può, se mai, favorire la trasformazione di acidi grassi liberi in trigliceridi.

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URINARY EXCRETION OF CAFFEINE AND ITS METABOLITES IN THE MOUSE

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Abstract—The excretion of radioactive metabolites in urine, feces, and expired air after the oral administration of 5 or 25 mg/kg of [^{3}H,^{14}C]caffeine to male CD-1 mice was investigated. Of the total radioactivity, 64–90 per cent was recovered in the urine. The major identifiable urinary metabolites were 1,7-dimethylxanthine, 3-methylxanthine, 7-methylxanthine, 1,3-dimethyluric acid and 1-methyluric acid. Of the administered caffeine, 3–6 per cent was recovered from the urine. Excretion of identifiable metabolites occurred rapidly and was 80 per cent complete in 8 hr following the 25 mg/kg dose. No consistent difference in urinary excretion of radioactivity was seen when mice fed caffeine since weaning were compared to those not previously exposed to caffeine, although the fecal excretory route was enhanced in the former.

DURING the past few years, much work has been performed on the physiological effects of caffeine in experimental animals. The fact that caffeine is a pharmacologically active agent and is consumed by the general population in the form of coffee and other beverages has made such work of especial interest. However, little has been published concerning the metabolism of caffeine by such experimental animals so as to determine if the metabolites in these species bear resemblance to those reported in man nor to what extent these metabolites may be responsible for the pharmacological effects observed with caffeine. Neither have reports appeared to indicate the kinetics of metabolism in these species so as to allow a comparison of tissue exposure.

Work with both the dog[1] and rat[2] has indicated that caffeine is readily absorbed after oral administration. However, little caffeine can be identified in the urine of the dog,[1] rabbit,[3] pig,[4] rat,[5] and man,[6,7] indicating extensive metabolism. The nature and the quantity of metabolites have only been looked at briefly. Cornish and Christman[6] and Schmidt and Schoyerer[7] have each looked at the excretion patterns of xanthines and, in the former case, uric acids, after caffeine administration in two human subjects. A striking disparity exists between the results of these two pairs of investigators. The first found the major xanthine metabolite to be 1-methylxanthine,[6] while the other authors identified theobromine and 1,7-dimethylxanthine as the major metabolites.[7] The report that 1-methyluric acid is the major metabolite in man[6] is corroborated by the finding of 1-methyl and 1,3-dimethyluric acid in the urine of a single rabbit and man after caffeine ingestion.[3] Unfortunately, extensive metabolism of this compound makes it difficult to interpret results reported in terms of total radioactivity after the administration of labeled caffeine.[8,9]

In the light of this paucity of published data, we have investigated in detail the metabolism of radioactively labeled caffeine in the mouse. The mouse was chosen for this initial study, since this species has been used extensively in studies of caffeine in the dominant lethal system.[10,11]*

MATERIALS AND METHODS

Labeled compounds. [^{14}C]Caffeine-1-methyl was obtained from Tracerlab Corp. with a specific activity of 4 mc/m-mole. Paper chromatography followed by radioautography in our laboratory indicated this material to be greater than 99 per cent homogeneous. Caffeine generally labeled with tritium was obtained from Amersham/Searle with specific activity of 1·5 c/m-mole. Radioautography revealed only one radioactive spot upon paper chromatography. These labeled compounds were diluted with caffeine and used together in a ratio of 1 μc ^{14}C/50 μc of tritium in our experiments.

Scintillation fluids. TEN solution is prepared from 3% naphthalene, 0·5% 2,5-diphenyloxazole, 0·01% 1*p*-bis [5–phenyloxazolyl[benzene (Pilot Chemical) in 55:45 toluene–ethyl cellosolve.† It has the advantage over many scintillation solvents of superior water solubility. Modified Bray's solution is standard Bray's solution[12] made 2·5% in 2-ethylhexanoic acid (Eastman) and 1·0% in ethanolamine (Fisher).† The modification allows carbonate in strong base to exist as a soluble salt and thus be readily quantitated.

Other materials. Theobromine, theophylline and uric acid used as standards were obtained from Matheson, Coleman & Bell. Caffeine was obtained from Eastman Organic Chemicals. AG-2-XS anion-exchange resin, 100–200 mesh, was obtained in the chloride form from Bio-Rad Laboratories. Other materials employed are described together with their application.

Dosage. Male CD-1 mice of approximately 30 g weight obtained from Charles River Breeding Laboratories were used throughout this study. Animals fed a diet of lab chow (Wayne) *ad lib.* were administered single oral doses of either 5 or 25 mg/kg of [^{3}H,^{14}C]caffeine by intubation of a 0·5- or 2·5- mg/ml solution containing 250 μc/ml of tritium and 5 μc/ml of ^{14}C. For the examination of the differential metabolism of a single dose of caffeine in animals chronically receiving caffeine, mice which had received 0·02 or 0·12 mg/ml of caffeine in their drinking water since weaning, at an average level of 4·1 or 25·2 mg/kg/day, and which were the third filial generation so treated, were given either 5 or 25 mg/kg of [^{3}H,^{14}C]caffeine respectively. Average chronic ingestion of caffeine was determined by measurement of water consumption during this 4-month period (time of weaning to time of dosing).

Sample collection. After the oral administration of [^{3}H,^{14}C]caffeine, groups of three animals each were placed in single Roth metabolism chambers.[13] Air was drawn continually into these chambers by vacuum pump, passing initially through Drierite calcium sulfate (W. A. Hammond Drierite Company) and then through Ascarite sodium hydrate asbestos absorbent (Arthur H. Thomas Company) before passing into the cage. The air drawn out of the chambers passed through a train of three towers each containing 100 ml of 8 N sodium hydroxide. The animals were provided with 375 mg/ml of "Get Up and Go" instant breakfast (Mead Johnson) *ad lib.* The system

* P. Thayer, in preparation.
† P. Barnowsky, personal communication.

was kept sealed except during the brief periods when samples were obtained. At these times, 0·1-ml samples of each of the tower contents, the urine diluted by the water necessary to wash the walls of the chamber, and the feces were removed. Collections of urine, feces and trap contents were made at 0·08, 0·5, 1, 2, 3, 5, 8, 24 and 32 hr after dosing with [^{3}H,^{14}C]caffeine. Samples were kept frozen until assayed.

Assay of radioactivity. Samples of the diluted urine and a 1 : 10 aqueous homogenate of feces were mixed with 10 ml TEN solution in scintillation vials. The 0·1-ml 8 N sodium hydroxide samples were diluted with 0·4 ml water and mixed with 10 ml of modified Bray's solution in vials.

Radioactivity was measured in a Nuclear Chicago Mark I scintillation spectrometer using external standard channel-ratio correction. Separate channels were optimized for detecting ^{3}H and ^{14}C emissions, and counting efficiencies were routinely determined. The quantitation of the two isotopes was effected by solution of the requisite simultaneous equation.

Location of ^{14}C radioactivity on developed paper chromatograms was accomplished by passing the paper strips through a Packard 4*II* radiochromatogram scanner using ^{14}C-ink (Schwarz BioResearch) as a marker. Quantitation was effected by excising areas of the chromatogram, folding them accordion-fashion, placing them in vials with 10 ml TEN, and using the scintillation spectrometer.

Chromatographic purification. Urine as obtained and 1 : 10 aqueous fecal hemogenates were applied to 0·5 × 8 cm (Pasteur pipette) AG-2-chloride anion-exchange columns which had been prepared by first washing the resin with one bed-volume of 0·1 N HCl and rinsing to neutrality with water. The columns were developed by batch elution; first with distilled water until radioactivity eluting from the column approached background, and then with 0·01 N HCl until the radioactivity of the eluate again approached background. The water eluate contained all the xanthines as well as other basic and neutral species and was referred to as the "non-acid fraction". The acid eluate contained the uric acids and other acidic compounds[6] and was called the "acid fraction". All eluates were flash evaporated to dryness.

Preparation of standards. Only caffeine, theobromine theophylline and uric acid standards were obtained commercially. Other standards were prepared by biological synthesis. An aqueous solution of caffeine was administered orally over 5 hr to an 83-kg man, resulting in a total dose of 1 g caffeine. Urine was collected over the subsequent 24 hr. Aliquots (50 ml) of this urine were subjected to column chromatography as described above, except that a 2·5 × 18 cm column was used and batch elution volumes were determined by monitoring the ultra-violet optical density of the eluate. The eluates were concentrated by lyophilization and chromatographed in various paper chromatographic systems. Specific paper chromatographic systems described in the literature for the separation of methylxanthines and methyluric acids were utilized. The migration of xanthines and uric acids in these systems was visualized by staining developed chromatograms using the two-step fresh mercuric acetate–diphenylcarbazone spray system of Dikstein *et al.*[14] Methyluric acids and the methylxanthines other than caffeine itself can be visualized by this method.

Those compounds developing color when sprayed were ascertained to be known methylated xanthines and uric acids by chromatography of larger amounts of the concentrated column eluates on Whatman 3MM paper. The areas of these developed chromatograms corresponding to areas which developed color with spray were excised

TABLE 1. IDENTIFICATION OF COMPOUNDS IN HUMAN URINE*

Suspected identity	Observed R_f	Reported R_f†	Observed absorption max. (nm)	Reported absorption max.‡ (nm)
1-Methyluric acid	0·42	0·42	291 (NaOH)	292
7-Methylxanthine	0·44	0·41	268 (HCl)	267
1-Methylxanthine	0·58	0·55	246 (NaOH)	243
			279	277
1,3-Dimethyluric acid	0·60	0·54	294 (NaOH)	294
1,7-Dimethylxanthine	0·68	0·66	265 (HCl)	262

* Chromatograms were developed with *n*-butanol–water–acetic acid, 4:1:1, and R_f values determined after the visualization of compounds using the mercuric acetate–diphenylcarbazone spray.[16] Spectra were taken of compounds eluted from developed chromatograms with either 0·01 N HCl or 0·05 N NaOH. These compounds were then employed as standards where required.

† Reference 14.

‡ Reference 6.

and eluted with either 0·1 N HCl or 0·05 N NaOH. Ultra-violet spectra were taken and compared to values reported in the literature (Table 1). These eluates were then used as secondary standards where required.

Based on the differential migration of standards, three chromatographic systems were selected for use in the identification of caffeine metabolites. These were: system I, *n*-butanol–water–acetic acid, 4:1:1, v/v;[6] system II, pyridine–25% aqueous ammonia, 94:6, v/v;[15] and system III, 95% ethanol–pyridine–water, 70:20:10, v/v.[15] All paper chromatograms were developed in the descending direction on Whatman No. 1 in. wide paper strips. Both standards and compounds obtained from human urine migrated in these systems in our hands with R_f values similar to those reported in the literature. Major differences were noted for system II in which most standards migrated with an R_f less than that reported and for uric acids in system III, which were also retarded.[15]

Identification of metabolites. The metabolites and other compounds obtained from the mouse which were eluted from the chromatographic columns were applied to Whatman No. 1 paper strips and developed in duplicate in system I. After chromatographic development and drying of the strips, the paper was cut into 1 × 2·5 cm pieces and placed in scintillation vials in the presence of 10 ml TEN scintillation solvent. The quantity of ^{3}H and ^{14}C at any R_f values was calculated using a computer program to evaluate the external standard counting efficiency and to solve the simultaneous equation. In later work it became apparent that those metabolites labeled with tritium alone were of minor significance. The developed paper chromatograms were thereafter scanned with the Packard radiochromatogram scanner which was adjusted such that tritium did not contribute to instrument response.

After the location of peaks had been determined by counting the paper pieces in scintillation vials, those areas corresponding to peak radioactivities were cut from the duplicate strip, eluted with water, spotted on a second Whatman No. 1 paper strip and developed in system II to aid further in identification. Only when results obtained with these two systems were questionable as to identification of metabolite were additional strips developed, peak materials eluted, and system III employed.

Three criteria were used in the identification of metabolites. Co-chromatography of radioactive materials with unlabeled standards (detected by ultra-violet light absorption) was the strongest evidence for the identification of an unknown. Second, the value of $^{3}H/^{14}C$ was helpful in corroborating identity, since it was known that the tritium was distributed between the ring nucleus of caffeine and the methyl groups whereas ^{14}C was only present as the 1-methyl group. Finally, the migration of a compound in accordance with published R_f values in multiple systems was used. Other radioactive compounds obtained which did not meet these criteria are described in this paper in terms of R_f values alone with only suggestions as to their possible identity.

RESULTS

In Fig. 1 are presented results on the eventual disposition of radioactivity after the administration of [^{3}H,^{14}C]caffeine, p.o., to male CD-1 mice. No large differences were observed between the disposition of a 5- and 25-mg/kg dose. The recovery of tritium parallels recovery of ^{14}C. A greater amount of tritium is found in the urine, presumably because some tritium eventually equilibrates with body water and is

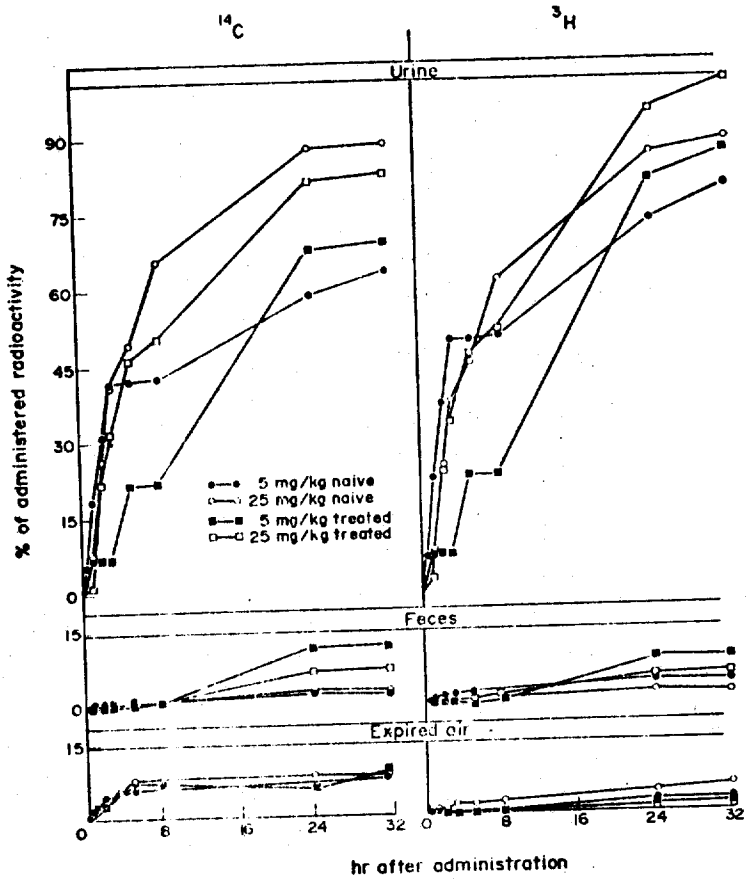

FIG. 1. Excretion of radioactivity after the administration of a single dose of [^{3}H,^{14}C]caffeine, p.o. Naive animals had not previously been exposed to caffeine. Treated animals had received an average of either 4·1 or 25·2 mg/kg/day caffeine since weaning.

excreted as ^3H_2O regardless of intermediary metabolism. At the later time periods, the tritium is indeed present as water, as shown later in this paper. More ^{14}C is recovered in the expired air than tritium. This, too, is to be expected, since the expired air route selectively disposes of carbon dioxide which can easily result from the metabolism of the 1-methyl group of caffeine after demethylation.

The only significant difference in disposition of a single dose of [^3H,^{14}C]caffeine between animals naive to caffeine as compared to those which had experienced daily caffeine ingestion from weaning is in the disposition by the fecal route. The percent of both tritium and ^{14}C excreted in the feces by animals ingesting caffeine daily was greater than twice and as much as 4·4 times the amount observed with naive mice. However, as to whether this is due to a change in the uptake of caffeine, an increased activity of caffeine-metabolizing organisms in the intestinal tract, or an increase in biliary secretion of caffeine metabolites has not been determined. As is shown later, little of the radioactive material which appears in the feces is caffeine.

The plateaus which are seen in the urinary excretion profile (Fig. 1) are best explained as resulting from the diuretic effect of caffeine. After the oral administration of either a 5- or 25-mg/kg dose, diuresis occurs. There follows an apparent compensation such that little urine is often produced at time periods from 1 to 8 hr. Since the animals are not catheterized and only that urine is collected which proceeds out the urethra, the plateaus seen in some of the curves reflect the decreased urine production during those time periods.

Metabolites in expired air. It was presumed that the ^{14}C trapped in the 8 N sodium hydroxide towers was present as $^{14}CO_2$ and that the tritium was present as water. This identification was further tested. Aliquots were obtained from each of the three towers connected for 32 hr to a Roth metabolism chamber containing three animals which had received a dose of 5 mg/kg [^3H,^{14}C]caffeine. The 10-ml aliquots were distilled in glass distilling apparatus. Samples (0·5 ml) of the distilled water recovered were dissolved in 10 ml TEN and counted. Less than 0·4 per cent of the administered ^{14}C dose could be accounted for in the distillate. Thus a maximum of 5 per cent of the ^{14}C present in the expired air is a volatile non-acidic compound.

The reverse experiment was also performed. Aliquots of the first tower were made acidic with concentrated HCl and then refluxed for 2 hr. At the end of that time, no ^{14}C-radioactivity could be found in the presence of the original high level of tritium, indicating that the ^{14}C present in the expired air after 32 hr following a 5-mg/kg dose is all acid volatile while the tritiated molecular species does not have a higher boiling point than that of water. This is consistent with $^{14}CO_2$ and ^3H_2O accounting for 95 per cent of the expired radioactivity.

Feces. The 1:10 fecal homogenates obtained from animals receiving 5 mg/kg [^3H,^{14}C]caffeine were centrifuged to determine if the radioactive material present exists in an insoluble form. A 0·05-ml sample of each homogenate was placed in a vial and dissolved in 0·5 ml of NCS solubilizer (Amersham-Searle) overnight before the addition of TEN and counting. The radioactivities of the centrifugal supernatant fraction and the whole homogenate at each time period were identical (within 10 per cent) such that it was possible to then subject this fraction alone to the usual column and paper chromatographic fractionation.

As presented in Table 2, "non-acidic" compounds predominated at early times with an increase in acid metabolites at later time periods. It can be seen from Table 2 that

TABLE 2. RECOVERY OF RADIOACTIVE COMPOUNDS FROM THE FECES

Fraction	Per cent administered dose of $^{14}C(^{3}H)$*						
	(0·5 hr)	(1 hr)	(2 hr)	(3 hr)	(5 hr)	(24 hr)	(32 hr)
Total radioactivity	0·35 (0·41)	0·80 (0·91)	0·51 (0·57)	0·11 (0·15)	0·23 (0·26)	2·20 (2·35)	0·05 (0·04)
Insoluble radioactivity	0·04 (0·02)	0·01 (0·06)	0·00 (0·00)	0·01 (0·01)	0·02 (0·02)	0·12 (0·00)	
Acidic compounds		0·19 (0·16)	0·13 (0·10)	0·02 (0·02)	0·10 (0·09)	0·68 (0·66)	
R_f 0·70 unknown		0·11	0·05	0·01	0·05	0·27	
1,3-Dimethyluric acid		0·06	0·04	0·01	0·04	0·31	
Non-acidic compounds		0·40 (0·56)	0·54 (0·35)	0·04 (0·06)	0·08 (0·11)	0·72 (1·12)	
Caffeine		0·17	0·16	0·01			
Feces/animal (g)	0·24	0·12	0·13	0·07	0·03	0·24	0·02

* Five mg/kg [^{3}H,^{14}C]caffeine, p.o., in three mice.

most of the material appearing in the feces was not identifiable as specific methylxanthines and methyluric acids. Whether this was due to the effect of bacterial action on known metabolites or to the presence of conjugates originating in the bile is not known. However, it is apparent that the fecal route was not a major excretory pathway for caffeine metabolites in the mouse and that apparently caffeine itself was almost entirely absorbed from the gastrointestinal tract and did not pass directly into the feces.

Urine. Many radioactive metabolites were found in the urine. Volatile radioactive constituents as well as methylxanthines and methyluric acids were demonstrable. The concentration of volatile materials was determined by placing urine samples in triplicate in scintillation vials. One scintillation vial of each grouping was made 0·01 N in HCl. The other two were left untreated. The acidified sample and one of the two remaining samples were allowed to air dry while the third was not. The comparison of the values obtained after adding scintillation solvent and counting in the scintillation spectrometer indicated the amount of volatiles and of acid-volatiles. For our purposes, the ^{14}C-acid-volatiles were considered to be CO_2, although exhaustive identification of the volatile constituents was not made. That tritium which was volatile with and without the addition of acid was considered to be water.

After a 25-mg/kg dose of [^3H,^{14}C]caffeine, 20 per cent of the tritium administered appears in the urine in a volatile form. Most of this tritium is probably present as water, but 6·6 per cent is volatile only when the urine is acidified. The tritiated water and other volatile ^3H materials are excreted more slowly than the xanthine and uric acid metabolites and presumably are the end products of metabolic action or result from exchange reactions which allow the incorporation of tritium into normal urinary compounds. Over 12 per cent of the ^{14}C administered as [^3H,^{14}C]caffeine appearing in the urine was also volatile. Of that a maximum of 1·5 per cent of the initial dose can be accounted for as CO_2. Most volatile ^{14}C radioactivity present in the urine first appeared in the 8-hr sample. The extent to which the 6·6 per cent dose of ^3H and the 10·7 per cent dose of ^{14}C correspond to one or more defined volatile species is not known.

The identity and amounts of nonvolatile urinary metabolites were determined by multiple chromatography as described in Methods. The actual results obtained with two acid fractions (Fig. 2) and two non-acid fractions (Fig. 3) are illustrated.

The large peak migrating 21 cm from the origin in the paper chromatogram of the acid fraction of the 1-hr urine sample (Fig. 2) was an unknown denoted as R_f 0·70 unknown as a result of its behavior in system I. The material migrating approximately 16 cm from the origin with the high ratio of ^{14}C to tritium was identifiable as 1,3-dimethyluric acid. The high ratio was the result of the elimination of the 8-hydrogen by oxidation to the uric acid and the fact that the 3-methyl group of the generally tritiated caffeine apparently contains little label. The small peak migrating approximately 12 cm from the origin was identifiable as 1-methyluric acid. The compound responsible for the peak preceding this, however, is not identifiable at the 1-hr time period.

The pattern observable in the acid fraction of the urine 24 hr after the administration of [^3H,^{14}C]caffeine was different from that at 1 hr. It is presented at the bottom of Fig. 2, slightly displaced from the 1-hr pattern above it. Thus the large peak migrating 18 cm from the origin was again 1,3-dimethyluric acid, this being the major con-

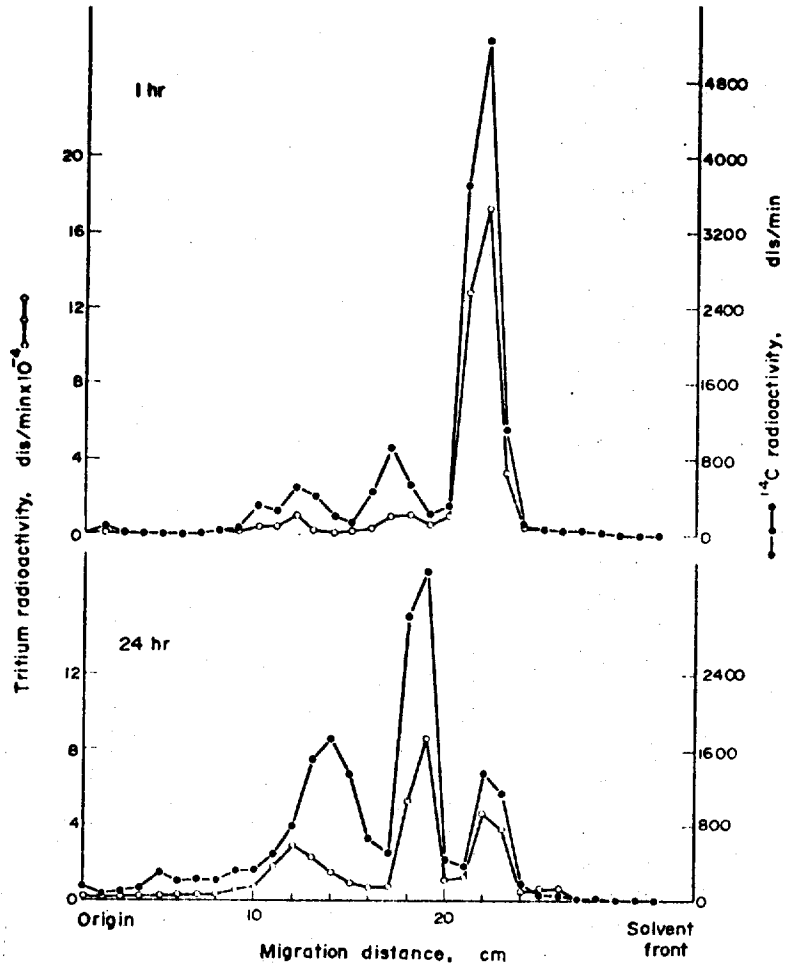

FIG. 2. Chromatographic distribution of radioactivity in the acid fraction of mouse urine at 1 and 24 hr after dosing. Urines were obtained from three male CD-1 mice at time periods after the administration of 25 mg/kg [^{3}H,^{14}C]caffeine, p.o., and the acid fractions were prepared as described in the text. Samples of these fractions were chromatographed on paper in system I. The developed chromatograms were cut into 1-cm segments and radioactivity was determined by scintillation counting.

stituent of the acid fraction at later times. Migrating further was the R_f 0·70 unknown, and just beyond this material was what may be a trace of 3,7-dimethyluric acid. The identity of this small amount of material, however, could not be confirmed. The material with the very high ratio of ^{14}C to tritium migrating 14 cm from the origin in this chromatogram was 1-methyluric acid and the tritium peak at 12 cm from the origin was 7-methyluric acid. The small ^{14}C peak at 5 cm from the origin is described as R_f 0·28 unknown, this being its normal R_f in system I.

In Fig. 3 are presented the analogous results for the non-acid fraction. Here the peak which migrates just behind the solvent front in the 1-hr urine sample was caffeine. The probable reason that the tritium and ^{14}C plots for caffeine did not coincide is

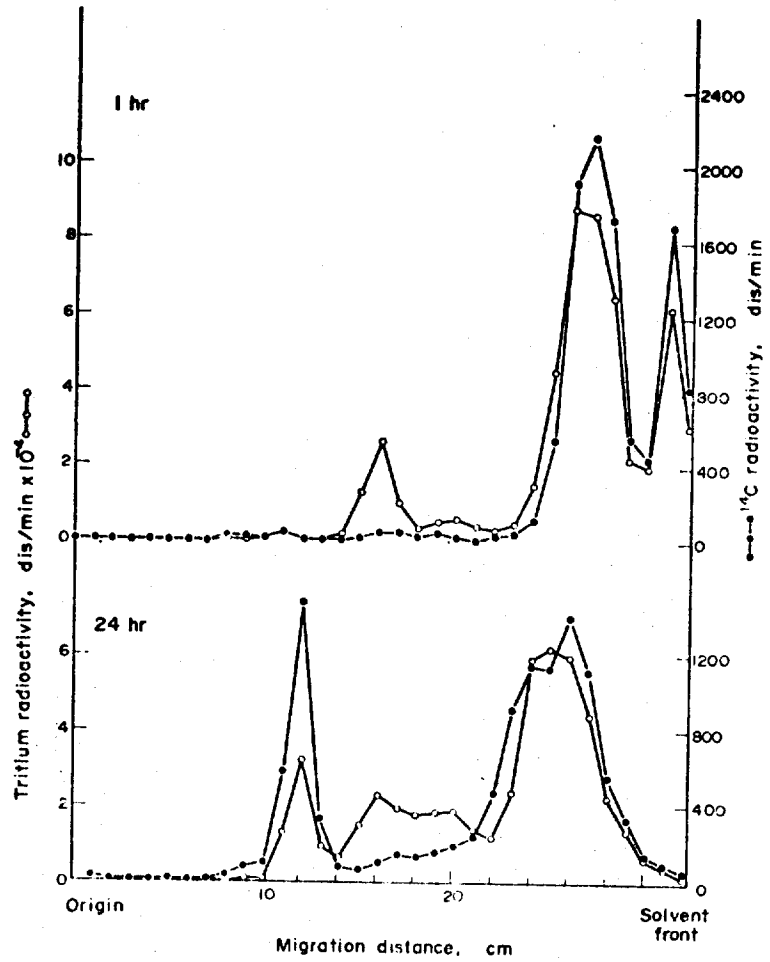

FIG. 3. Chromatographic distribution of radioactivity in the non-acid fraction of mouse urine at 1 and 24 hr after dosing. Urines were obtained from three male CD-1 mice at time periods after the administration of 25 mg/kg [^{3}H,^{14}C]caffeine, p.o., and the non-acid fractions were prepared as described in the text. Samples of these fractions were chromatographed on paper in system I. The developed chromatograms were cut into 1-cm segments and the radioactivity in each segment was determined by scintillation counting.

that some tritium has been lost from the caffeine by exchange in the body. The ratio of ^{14}C to tritium in caffeine is similar to that observed for the R_f 0·70 unknown in Fig. 2. The large peak centered at a migration distance of approximately 28 cm from the origin was a complex peak which was only resolved by subsequent chromatography. The leading edge contained a trace of what might have been theophylline, but was most especially an unknown compound, termed R_f 0·93 unknown, from its behaviour in system II. The trailing edge of this peak, which had a lower ratio of ^{14}C/^{3}H, was 1,7-dimethylxanthine. The very slight peak composed of tritium alone which has migrated 20 cm from the origin was 3-methylxanthine and the larger peak with a migration distance of 17 cm was 7-methylxanthine.

A different chromatographic pattern was found for the non-acid fraction of the 24-hr urine sample obtained from mice after a 25-mg/kg [^{3}H,^{14}C]caffeine dose (Fig. 3). In the urine collected 8 and 24 hr after the administration of [^{3}H,^{14}C]caffeine, no caffeine was detectable. Some 1,7-dimethylxanthine was still present, having migrated 26 cm from the origin. Just behind (22–25 cm) the 1,7-dimethylxanthine there was evidence for two compounds, one with a ^{14}C/^{3}H ratio of <1 and one existing as a shoulder on that peak with a ratio of >1. The identity of this material was not resolved by chromatography in other systems. 3-Methylxanthine and 7-methylxanthine again were identifiable, but this time contaminated by some ^{14}C-containing material. The large sharp peak which had migrated 12 cm from the origin was not identifiable as a methylated xanthine and has been denoted as R_f 0·29 unknown, based on its relative migration characteristics in system I. It is apparent from Fig. 3 that no 1-methylxanthine was present at either of the two time periods shown, as this material would have existed as a ^{14}C-containing peak almost halfway between the peaks identified as 3-methylxanthine (20 cm) and 1,7-dimethylxanthine (26 cm).

Based on the chromatographic evidence presented in Figs. 2 and 3, and through the use of the migration characteristics of compounds in systems II and III, it is possible to identify and quantitate the urinary radioactive metabolites occurring in the urine of the CD-1 mouse after the administration of a 5- or 25-mg/kg dose of [^{3}H,^{14}C]caffeine. The results of such an analysis for the three mice given a 25-mg/kg dose of radioactive caffeine are presented in Fig. 4. This figure details the presence of metabolites in urine expressed as a per cent of administered radioactivity versus time.

It can be readily observed that in this case only 6 per cent of the caffeine administered passes into the urine. A greater amount of 1,7-dimethylxanthine, 1,3-dimethyluric acid, and 1-methyluric acid was found (11, 10·5 and 8·5 per cent respectively). It is apparent that the methyluric acids continue to appear in the urine after 24 hr following caffeine administration, whereas the xanthines have mostly been excreted at shorter time periods. The quantities of 3-methylxanthine and 7-methylxanthine excreted are somewhat tenuous, since the extent to which the tritium within these compounds has been lost is not known. The fact that both tritium and ^{14}C are expressed as per cent of administered radioactivity allows a cumulative recovery of material greater than that expressed as total radioactivity of either tritium or ^{14}C alone.

As can be determined from Fig. 4, the second most abundant radioactive compound appearing in the urine was the material denoted as the R_f 0·70 unknown. This material was eluted from the column as an acid and was found in samples of both mouse urine and mouse feces as well as in monkey urine (work in progress). It had the highest R_f of all acid fraction radioactive species. As seen in Fig. 4, the contribution of radioactivity associated with this compound declined relative to the rest of the sample with increasing time. Thus, although after 1 hr it made up approximately 50 per cent of the acid fraction, in the 24-hr sample it accounted for only 5 per cent. Initially it was thought that this material might be 1,3,7-trimethyluric acid based on its hydrophobic nature. However, when it was eluted from system I and run in system II, two peaks resulted. The major one of these had a low R_f (0·18), indicating that the compound became more hydrophilic in base. The second peak (R_f 0·90) represented 20 per cent of the material. Subsequent rechromatography revealed that the R_f 0·90 peak was a breakdown product of the R_f 0·18 material. Thus the best indications are that the

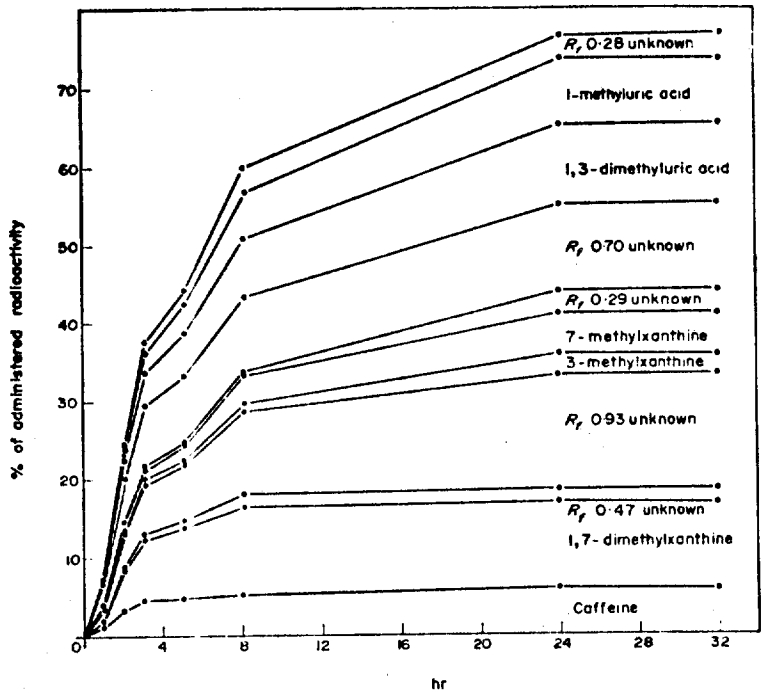

FIG. 4. Major radioactive metabolites in the urine of the mouse after the administration of 25 mg/kg [^{3}H,^{14}C]caffeine, p.o. Urine of three male CD-1 mice was pooled at each time period. The identity and quantity of metabolites were determined by multiple chromatography as described in the text. Data are presented as summated cumulative excreted per cents of administered radioactivity.

unknown identified as R_f 0·70 unknown was an acidic compound which was especially hydrophobic in acid and which was somewhat unstable. It was not a methyluric acid.

Those compounds identified in Fig. 4 as the R_f 0·93 and R_f 0·47 unknowns are denoted by the R_f values which these compounds demonstrate in system II. In system I, they co-chromatograph with 1,3-dimethylxanthine and 1,7-dimethylxanthine. Based on their behaviour in system I, it was presumed that they shared solubility characteristics with the dimethylxanthines. However, the hydrophobicity of the R_f 0·93 unknown in system II dispels this idea. Neither the R_f 0·93 unknown nor the R_f 0·47 unknown could be identified as any known methylxanthine. It was further demonstrated that the two major unidentified metabolites, the R_f 0·70 and R_f 0·93 unknowns, did not co-chromatograph with authentic carbonate, urea, methionine, thymidine or any of the 15 amino acids of an algal protein hydrolysate.

The R_f 0·29 unknown eluted with the acid fraction and the R_f 0·28 unknown eluted with the non-acid fraction were both minor metabolites. Based on their hydrophilicity and later appearance in the urine they were presumed to be further degradation products of caffeine still maintaining the labeled 1-methyl carbon. As such, these compounds could be any common excretory product in which this particular carbon had been reincorporated by the metabolic pathways of the mouse.

In addition to those compounds shown in Fig. 4, traces of other constituents were found. Less than 1 per cent of a compound which might be theophylline is present in

urine through 3 hr after the administration of caffeine. Less than 0·1 per cent of material possibly identifiable as theobromine has been observed. This latter material was first detected 8 hr after administration of caffeine, in contrast to the earlier appearance of the other methylxanthines. The identification of both dimethylxanthines is questionable as a result of the small amount of compound present in the urine. Less than 0·5 per cent of the administered ^{14}C radioactivity in both the acid and non-acid fractions remains at the origin during chromatography in system I.

The pattern of metabolites in the urine after administration of a 5-mg/kg dose was similar to that reported (Fig. 4) after a 25-mg/kg dose. The percentage of caffeine, however, was only half that seen with the 25-mg/kg dose and it appeared more quickly in the urine, with less than 0·1 per cent appearing in the urine after 3 hr. No large differences in the amount of the other non-acid fraction metabolites found in the urine after the 5-mg/kg caffeine dose were observable. More than two-thirds of these materials were excreted within 8 hr after dosing as was all of the caffeine. For comparison, the total amount of acid fraction metabolites appearing in the urine over 32 hr after a 5-mg/kg dose, expressed as a percentage of the administered ^{14}C radioactivity, was 3·0, 8·2, 4·4 and 9·0 for the R_f 0·28 unknown, 1-methyluric acid, 1,3-dimethyluric acid and the R_f 0·70 unknown, respectively. Only the dimethyluric acid value was at variance with the values obtained after a 25-mg/kg dose (Fig. 4). No attempt was made to identify the metabolites produced by those mice which were given [^{3}H,^{14}C]-caffeine after having received caffeine in their drinking water since weaning.

DISCUSSION

The chronic administration of caffeine to mice did not noticeably alter the respiratory excretion of either a 5- or 25-mg/kg oral dose of [^{3}H,^{14}C]caffeine. The same percentage and rate of $^{14}CO_2$ production was observed with both doses and in both chronic and naive animals, indicating that a 1-methyldemethylase is not induced by chronic administration of caffeine or inhibited by high concentrations of caffeine, as has been suggested.[16] An increased amount of fecal radioactivity was observed in chronically treated animals, but the feces still remained a minor excretory route. No consistent difference in total urinary excretion of radioactive metabolites was demonstrable.

A difference in the percentage of radioactivity excreted in urine between those animals receiving the 5- and 25-mg/kg dose was obtained. A greater percentage of administered radioactivity (both ^{3}H and ^{14}C) was excreted after a 25-mg/kg dose. Presumably catabolic reactions allow some caffeine-derived radioactivity to be incorporated into normal body constituents which remain in the carcass. This amount of incorporation probably remains relatively constant whether a 5- or 25-mg/kg dose is given. This can explain the higher recovery of total radioactivity on a percentage basis after the 25-mg/kg dose.

In these experiments, a slightly greater percentage of caffeine also appeared in the urine after 25 mg/kg of oral caffeine. The male CD-1 mouse excretes 6 per cent of a 25-mg/kg oral caffeine dose as caffeine but only about 3 per cent as caffeine after a 5-mg/kg dose. This compares with the reported 0·5–1·5 per cent recovery of caffeine in the urine of man,[1,6,7] 2 per cent in rat,[5] 6 per cent in pig[4] and 13·3 per cent in rabbit urine.[3]

The mouse did not excrete 1-methylxanthine, as does man,[6,7] nor the theobromine

reported by Schmidt and Schoyerer.[7] However, 1,7-dimethylxanthine and 7-methylxanthine are produced in greater quantity by the mouse than by man, 11 and 5 per cent compared to 0·2–0·7 per cent and 0·4–0·9 per cent in man.[6] No measurable theophylline was observed with either species and the 3-methylxanthine found in mouse urine has not been reported for man.[6,7] Increased uric acid excretion after caffeine ingestion would not have been observed by our method, since none of the labeled atoms incorporated into our [^{3}H,^{14}C]caffeine remain in uric acid. The ^{14}C-methyl label would be lost by demethylation and the general tritium label would be lost by demethylation and oxidation. None of the four hydrogen atoms in uric acid derive from caffeine.

1-methyluric acid has been reported to be the major metabolite resulting from caffeine ingestion in man, accounting for 25–30 per cent of a 1 g oral dose.[6] In the mouse, 1-methyluric acid accounts for only 8 per cent of the administered caffeine radioactivity after either a 5- or 25-mg/kg dose. The absence of 1-methylxanthine and decreased production of 1-methyluric acid probably indicate the presence of a more active 1-methyldemethylase in the mouse. Differences in activity of such an enzyme would help explain the decreased recovery of identifiable xanthines and uric acids in the mouse; less than 44 per cent compared to 62–75 per cent in man.[6]

In rabbit urine, 1-methyluric acid plus 1,3-dimethyluric acid accounted for 25 per cent of the administered caffeine.[3] Thus, the CD-1 mouse, with a combined total of 13–21 per cent for these two metabolites, is more like the rabbit in this regard than like man (35–40 per cent). However, with the exception of 1 methylxanthine, the differences in urinary metabolites produced by the CD-1 mouse and man relate to quantity and not identity.

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TISSUE DISTRIBUTION OF CAFFEINE AND ITS METABOLITES IN THE MOUSE

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Abstract—The disposition of a single oral dose of 5 or 25 mg/kg of [^{3}H, ^{14}C]caffeine in the brain, heart, kidneys, liver, lungs, spleen, testes, muscle, plasma and erythrocytes of the CD-1 mouse was investigated. Caffeine appeared in all tissues within 5 min. It distributed in proportion to tissue water only at 1 hr after dosing, entering and leaving various organs at different rates. The maximum organ half-life of caffeine was 5 hr. A shorter half-life was observed after the 5-mg/kg dose, but the disposition of both doses was otherwise similar. 1,7-Dimethylxanthine is the major tissue metabolite. Little methyluric acid was present in the tissues. No methylxanthine nucleotides were demonstrable in the liver. The exposure of organs to caffeine and 1,7-dimethylxanthine was calculated.

THE EFFECT of caffeine and theophylline on isolated tissue and cellular systems has been the subject of much investigation, expecially since the advent of interest in the mediating role of cyclic adenosinemonophosphate (c-AMP). These methylxanthines have been studied as to effects on perfused organs, tissue homogenates, tissue-cultured cells and a variety of nonmammalian test systems *in vitro*. The relevancy of any of this work to possible effects to be expected in the situation *in vivo* is dependent upon the extent to which any organ is perfused or contains caffeine and theophylline *in vivo* after oral administration. The actual exposure of individual tissues may differ widely.

Although work with the rat indicates that caffeine is poorly absorbed through the stomach,[1] Axelrod and Reichenthal[2] have shown that upon ingestion, it is completely absorbed from the gastrointestinal tract within 1 hr in man and distributes in the tissues of the dog in direct proportion to tissue water. A similar distribution between plasma and testes or ovaries within 2 hr after i.v. administration has been observed in man.[3] Subsequently, the plasma level falls to half its value in 3·5 and 5 hr in man and dog respectively.[2] A half-life of approximately 13 hr for caffeine in the plasma, liver, muscle and kidney (and 20 hr in the back fat) of the pig has been found.[4] A shorter serum half-life is reported for the rat with neither caffeine nor metabolites demonstrable 5 hr after dosing.[5] These findings represent the available reported data on the disposition of caffeine in mammalian tissues.

Techniques have been developed for the identification of metabolites of caffeine.[6] In the work reported here, these techniques have been applied to a complete study of the physiological disposition of caffeine in the mouse in order to determine the importance of the concentration and time of contact of caffeine metabolites within the tissues in relation to tissue levels of caffeine itself, and to the caffeine administered.

MATERIALS AND METHODS

Materials. Sodium heparin was a product of Riker Laboratories. Precoated cellulose thin-layer plates containing fluorescent indicator were purchased from Brinkmann. Analytical grade AG-1-X8 formate resin, 200–400 mesh, was obtained from Bio-Rad Laboratories; it was washed first with 6 N formic acid–1 N sodium formate and then with water before use. All other reagents were obtained from sources reported previously.[6]

Dosage. Male CD-1 mice of approximately 30 g weight obtained from Charles River Breeding Laboratories were used throughout this study. Male mice were used, since they have been the subject of previous experiments investigating the effects of caffeine.[7] Animals raised on a diet of lab chow (Wayne) were given single oral doses of either 5 or 25 mg/kg of [^{3}H,^{14}C]caffeine by intubation of a 100 g/ml solution in which the ratio of tritium to ^{14}C was 15·6:1. The large ratio decreased the opportunity for errors due to dual label counting and calculation.

Groups of three animals were used for each time period and each dose. Animals were sacrificed by cervical dislocation at 0·08, 0·5, 1, 3, 8 and 24 hr after dosing, and the brain, heart, kidneys, liver, lungs, spleen and testes were removed by immediate dissection. A muscle sample was excised from muscle adjacent to the femur. Whole blood was obtained by heart puncture with a heparinized hypodermic syringe.

For the radioautography experiment, 8-month-old male CD-1 mice which had received 0·02, 0·06 or 0·12 mg/ml of caffeine in their drinking water since weaning, estimated to correspond to 4·1, 12·2 or 25·2 mg/kg/day, were given a single 10-mg/kg dose of 1-methyl [^{14}C]caffeine. Twenty-four hr later the mice were sacrificed by immersion in a hexane–dry ice mixture. The frozen carcasses were stored at −26° for microtomy.

Tissue preparation. Whole blood was separated into plasma and red blood cells by centrifugation in an International desktop centrifuge. Erythrocytes and all other tissues were homogenized using a Potter–Elvehjem homogenizer to yield a 9% suspension in normal saline. Samples (0·05 ml) of homogenate were hydrolyzed overnight with 0·5 ml of Amersham/Searle NCS solubilizer and counted in TEN solvent[8] in the Nuclear Chicago Mark I scintillation spectrometer.

Purification. For the effective recovery of metabolites, extraction was required. Although less than 20 per cent of a 60 mM caffeine, theophylline or theobromine solution is bound by 2% bovine serum albumin, 83 per cent of 1,3,7-trimethyluric acid is so bound.[9] The samples of homogenate were therefore mixed with an equal volume of 0·4 M perchloric acid so as to extract all such bound compounds. The precipitate resulting was collected, centrifuged and washed twice with a half-volume of 0·2 M perchloric acid before being discarded. The washes and the original supernatant were combined and neutralized with 5 M potassium hydroxide. Potassium perchlorate was removed by centrifugation. The acid extract was then applied to a 0·5 × 8 cm AG-2-chloride column and the column was developed as reported in the previous paper.[6]

Quantitation. The acidic and non-acidic radioactive metabolites present in the column eluates were identified by paper chromatography in multiple chromatographic systems.[6] The location of any ^{14}C-metabolite in a developed chromatogram was determined by scanning with a Packard radiochromatogram scanner. Quantitation was then effected using a Keuffel and Esser compensating polar planimeter to determine

the areas of peaks appearing on the scanner recording ratemeter chart output. The ratio of any peak area to the total area obtained by scanning the entire chromatogram was assumed to be proportional to the ratio of the radioactivity of the specific compound responsible for the peak to the total radioactivity of the sample applied. This assumption was shown to be correct for the quantitation of metabolites present in urine.

Nucleotide analysis, Livers obtained from mice at 1, 3 and 8 hr after p.o. administration of [^{3}H,^{14}C]caffeine at 25 mg/kg and at 3 hr after a 5-mg/kg dose were analyzed. The chromatographic method used was a modification of the gradient elution technique of Chandhuri *et al.*[10] The perchloric acid-soluble fraction of the livers was loaded on to AG-1-formate anion-exchange columns (18 × 0·5 cm) and were eluted with: (1) 20 ml of H_2O; (2) 80 ml of 0·05 M formic acid; (3) approximately 220 ml of an asymptotic formic acid gradient, with 4 M formic acid in the reservoir and 100 ml of H_2O in the mixing chamber; (4) 50 ml of 2·0 M ammonium formate. Fractions of 3 ml were collected and the radioactivity in each fraction was determined.

To determine if any of the peaks eluted by the formic acid gradient were nucleotides, fractions comprising each potential nucleotide peak were pooled, evaporated to dryness and were taken up into 3·0 ml of 0·1 M glycine buffer, pH 10·5. To one-half of this solution, 10 μl of alkaline phosphatase was added, and to the other half, 10 μl water was added. The mixtures were incubated at 34° for 2 hr. Preliminary experiments had shown that under these conditions 9 μmoles of inosinic acid was converted to inosine. The reaction mixtures were evaporated to dryness, taken up into water, and desalted by passing through Dowex-50 cation-exchange resin. The eluate was dried, redissolved in water, and applied to precoated cellulose plates. Thin-layer chromatography was finally used to determine if alkaline phosphatase altered the chromatographic mobility of the suspect radioactive materials.

Acid-insoluble radioactivity. Samples of the acid-insoluble fraction of livers were extracted twice with 95% ethanol and once with 95% ethanol–ether, 3:1. The residues were suspended in 0·3 M potassium hydroxide and centrifuged to remove the insoluble potassium perchlorate. The radioactivity in the ribonucleic acid (RNA) extract was determined in TEN scintillator solution. The acid-insoluble precipitate was extracted twice with 0·5 N perchloric acid at 90° for 15 min. The extracts were combined and neutralized with KOH and centrifuged to remove the insoluble potassium perchlorate. The radioactivity in the resulting deoxyribonucleic acid (DNA) extract was determined in TEN scintillator solution.

RESULTS

The concentrations of caffeine, its major metabolites and of unidentified radioactive species within the organs and tissues of the mouse after oral administration of [^{3}H,^{14}C]caffeine are presented in Tables 1, 2 and 3. The values for organs are expressed as the per cent of the administered ^{14}C present in the whole organ. The values for tissues have been calculated for the total muscle mass based on the per cent of muscle present in man,[11] and for the total body complement of blood plasma and erythrocytes based on the volumes reported in the literature for the mouse.[12] The results presented are the combined data obtained with three animals at each time period.

It is apparent that caffeine radioactivity rapidly entered these organs and tissues within 5 min after administration of a 25- or 5-mg/kg (Tables 1 and 3) oral dose.

TABLE 1. MAJOR ^{14}C-COMPOUNDS IN THE ORGANS OF THE MOUSE WITHIN 1 hr AFTER A 25-mg/kg DOSE OF [^{3}H,^{14}C]CAFFEINE

Compounds	Per cent of administered ^{14}C-radioactivity in									
	Brain (0·40 g)*	Heart (0·15 g)	Kidneys (0·47 g)	Liver (1·70 g)	Lungs (0·23 g)	Spleen (0·17 g)	Testes (0·22 g)	Muscle (12·9 g)	Blood plasma (1·46 ml)	Erythrocytes (0·87 ml)
At 5 min										
Total radioactivity	0·63	0·31	1·18	7·2	0·56	0·48	0·14	13·1	4·1	0·52
Extractable radioactivity	0·65	0·31	1·17	6·7	0·58	0·50	0·14	13·1	4·1	
Total acids	0·01	0·00	0·02	0·06	0·00	0·01	0·00	0·66	0·06	
Total non-acids	0·58	0·28	1·20	5·5	0·48	0·45	0·13	12·5	2·82	
Caffeine	0·58	0·28	1·20	5·5	0·48	0·45			2·13	
At 30 min										
Total radioactivity	0·94	0·42	1·54	5·1	0·61	0·46	0·67	29·9	4·2	0·69
Extractable radioactivity	0·97	0·47	1·49	5·8	0·65	0·49	0·67	29·4	3·5	
Total acids	0·01	0·01	0·04	0·25	0·01	0·01	0·01	0·49	0·18	
1-Methyluric				0·12						
Total non-acids	0·88	0·37	1·37	5·5	0·64	0·36	0·54	30·9	2·94	
Caffeine	0·65	0·27	1·01	4·8	0·52	0·28	0·54	23·5	2·07	
1,7-CH_3-xanthine	0·23	0·10	0·35	0·65	0·12	0·08			0·69	
At 1 hr										
Total radioactivity	0·87	0·40	2·02	5·9	0·56	0·43	0·95	35·8	4·8	0·62
Extractable radioactivity	0·85	0·38	1·89	5·8	0·58	0·52	1·21	3·52		
Total acids	0·01	0·01	0·11	0·41	0·01	0·02	0·14	0·00		
1-Methyluric			0·04	0·24	0·00	0·00				
Total non-acids	0·70	0·33	1·69	4·7	0·51	0·39	0·60	34·0		
Caffeine	0·61	0·26	0·98	2·7	0·38	0·25	0·38	22·7		
1,7-CH_3-xanthine	0·09	0·07	0·49	1·6	0·04	0·14	0·22			

* The average weight of each organ is given in parentheses.

TABLE 2. MAJOR ^{14}C-COMPOUNDS IN THE ORGANS OF THE MOUSE 3 hr AFTER A 25-mg/kg DOSE OF [^{3}H,^{14}C]CAFFEINE

Compounds	Per cent administered ^{14}C-radioactivity in									
	Brain (0·37 g)*	Heart (0·15 g)	Kidneys (0·47 g)	Liver (1·50 g)	Lungs (0·23 g)	Spleen (0·16 g)	Testes (0·25 g)	Muscle (12·9 g)	Blood plasma (1·46 ml)	Erythrocytes (0·87 ml)
At 3 hr										
Total radioactivity	0·32	0·19	1·82	3·9	0·30	0·26	0·27	13·4	2·07	0·42
Extractable radioactivity	0·35	0·19	1·65	3·6	0·28	0·21	0·26	16·9	2·07	
Total acids	0·01	0·01	0·24	0·53	0·02	0·02	0·02	1·17	0·33	
1-Methyluric			0·11	0·36	0·00	0·01			0·15	
Total non-acids	0·31	0·15	1·42	2·4	0·19	0·17	0·21	13·4	1·44	
Caffeine	0·22	0·08	0·47	1·14	0·12	0·10	0·12		0·69	
1,7-CH_3-xanthine	0·09	0·06	0·53	0·90	0·08	0·08	0·09		0·42	
At 8 hr										
Total radioactivity	0·04	0·03	0·33	1·15	0·10	0·08	0·14	6·6	0·60	
Extractable radioactivity	0·06	0·03	0·26	0·86	0·08	0·05	0·16	6·6	0·57	
Total acids		0·00	0·03	0·19	0·01	0·02	0·03			
1-Methyluric			0·02	0·17						
Total non-acids		0·02	0·19	0·44	0·03	0·03	0·09			
At 24 hr										
Total radioactivity	0·03	0·02	0·22	0·60	0·07	0·05	0·03	1·47	0·24	0·07
Extractable radioactivity	0·06	0·01	0·12	0·28	0·07	0·03	0·05	1·47	0·24	
Total acids		0·00			0·00	0·01	0·00			
Total non-acids		0·01			0·01	0·02	0·01			

* The average weight of each organ is given in parentheses.

TABLE 3. MAJOR ^{14}C-COMPOUNDS IN THE ORGANS OF THE MOUSE AFTER A 5-mg/kg DOSE OF [^{3}H,^{14}C]CAFFEINE

Compounds	Per cent administered ^{14}C-radioactivity in									
	Brain (0·37 g)*	Heart (0·15 g)	Kidneys (0·47 g)	Liver (1·77 g)	Lungs (0·26 g)	Spleen (0·17 g)	Testes (0·24 g)	Muscle (12·9 g)	Blood plasma (1·46 ml)	Erythrocytes (0·87 ml)
At 5 min										
Total radioactivity	0·57	0·31	1·06	6·6	0·65	0·60	0·17	21·2	3·54	0·60
Extractable radioactivity	0·56	0·28	1·06	6·7	0·44	0·64	0·17	20·4	3·46	
Total acids	0·01	0·01	0·02	0·10	0·01	0·00	0·00	0·29	0·15	
Total non-acids	0·48	0·23	1·11	5·7	0·41	0·53	0·14	17·4	2·54	
Caffeine	0·41	0·23	1·01	5·7	0·41	0·53		17·4	2·54	
1,7-CH_3-xanthine			0·10							
At 1 hr										
Total radioactivity	0·58	0·29	2·40	6·9	0·73	0·29	0·32	23·8	4·52	0·58
Extractable radioactivity	0·58	0·36	2·38	6·4	0·47	0·32	0·36	23·4	4·43	
Total acids	0·01	0·02	0·19	0·62	0·20	0·01	0·02	0·73	0·63	
1-Methyluric					0·02					
Total non-acids	0·54	0·28	2·24	5·4	0·36	0·27	0·31	20·6	3·09	
Caffeine	0·25	0·14	0·85	1·63	0·19	0·16	0·18	12·7	0·86	
1,7-CH_3-xanthine	0·10	0·14	1·27	1·50	0·17	0·11	0·13	8·0	1·06	
At 8 hr										
Total radioactivity	0·04	0·04	0·35	2·0	0·20	0·10	0·08	3·6	0·60	0·10
Extractable radioactivity	0·09	0·03	0·24	1·2	0·11	0·05	0·09	5·1	0·40	
Total acids	0·01	0·01	0·03	0·18	0·01	0·00	0·01	0·55		
1-Methyluric										
Total non-acids	0·04	0·02	0·18	0·76	0·03	0·03	0·06	2·3		
1,7-CH_3-xanthine								2·3		

* The average weight of each organ is given in parentheses.

It is also apparent from the seven organs for which the data are given that caffeine was responsible for almost all of the radioactivity present at this early time. All of the radioactivity was extractable. At this early time period, very little radioactivity was present in the acid fraction of the extracted tissue and only in plasma were acid metabolites observable. These compounds were present in low concentration and have not been identified.

Thirty min after administering the 25-mg/kg dose, the total radioactivity of the organs and tissues had increased over the 5-min level, but not by a very large margin. The amount of caffeine, expressed as a per cent of the administered dose, remained relatively constant within the organs. Practically all of the radioactive material present at the 30-min time period was extractable. 1,7-Dimethylxanthine became apparent here as the major metabolite. A non-acid fraction material with the chromatographic property of a dimethylxanthine was also present in low concentration in muscle and plasma. The acid fraction contained low concentrations of three hydrophobic compounds which were not identified. 1-Methyluric acid was the only identifiable acid metabolite present in measurable amount. A trace of 1,3-dimethyluric acid may have existed in certain organs. The difference between extractable radioactivity and the sum of total acids plus total non-acids indicates the presence of volatile constituents. This was especially noticeable in the blood plasma.

By 1 hr after the administration of [^{3}H,^{14}C]caffeine, the concentration of caffeine in most organs accounted for only half of the total radioactivity. 1,7-Dimethylxanthine remained as the major metabolite. The ratio of 1,7-dimethylxanthine to caffeine varied widely between organs, but at no time did the caffeine metabolite exceed caffeine in concentration after the 25-mg/kg dose and only rarely after the 5-mg/kg dose. At this time, the highest recovery of radioactivity from these organs was affected; 52 per cent of the administered dose. Since only about 10 per cent of the administered dose had been excreted by this time,[6] the remaining 38 per cent of the radioactivity was presumably in the 38 per cent of the body weight unsampled in this study, i.e. the bladder, fat bodies, intestines.

Table 2 and the 8-hr data of Table 3 reflect the presence of radioactive materials within the organs of the mouse at later time periods. Besides caffeine, 1,7-dimethylxanthine and 1-methyluric acid, after 3 hr, a material which may have been 1,7-dimethyluric acid was present in liver, lungs, kidneys, testes and plasma. However, as can be determined by comparing the total acid fraction at this time to the dose administered, only a very small amount of radioactivity can exist as this metabolite. By 3 hr after dosing, considerable material in the metabolically active organs, such as the liver, spleen and kidneys, existed as nonextractable radioactivity. This amount of nonextractable material increased through 8 and 24 hr as a percentage of the total radioactivity at each time. Nonextractable ^{14}C-radioactivity presumably results from the reincorporation of the radioactive methyl group into insoluble body constituents such as protein.

By the 8-hr and 24-hr time periods, the level of radioactivity had become so low as to make it difficult to identify metabolites. 1-Methyluric acid was present in certain organs. Its apparent absence in other organs, however, may be due to the sensitivity limitations of our method of analysis. The absence of specific xanthine and other non-acid metabolites at these last two sampling times is due to the low level of radioactivity available in the organs at this time for chromatographic identification. By 24 hr, it is

presumed that the 0·01–0·03 per cent of the administered dose of ^{14}C-radioactivity which remained as acid or non-acid metabolites within the organs was present not as xanthines or uric acids, but as normal cellular compounds.

The disposition in the organs of the tritium administered paralleled that for ^{14}C. The per cent of tritium as total radioactivity, extractable radioactivity, total acids and total non-acids throughout the early time periods was as shown in Tables 1 and 3. However, at the 8-hr and 24-hr time periods, the ratio of tritium to ^{14}C present as total or extractable radioactivity increased greatly. The total tritium recovered after 8 hr represented 20 per cent of the administered dose. It could be presumed that much of this tritium was present as body water resulting from tritium exchange with the generally labeled caffeine molecule and from labeled metabolites. Such water would not have been selectively excreted as were the ^{14}C metabolites.

In Table 3 are presented the results obtained after the administration of a 5-mg/kg dose of [^{3}H,^{14}C]caffeine at three selected time periods. Since the data are presented as a percentage of radioactive dose administered and not in terms of concentration, the data are directly comparable to those presented in Tables 1 and 2. It may be observed that the 5 mg/kg dose was distributed within the organs of the mouse and as defined metabolites in a manner similar to that for the 25-mg/kg dose. In percentage terms, however, the 5-mg/kg dose was metabolized somewhat more rapidly. This can be adjudged by comparing the 1-hr values for caffeine presented in Table 3 to those in Table 1. In absolute terms, of course, a greater number of micrograms was metabolized within the same time period after the 25-mg/kg dose.

The disposition of trace metabolites after the 5-mg/kg dose of [^{3}H,^{14}C]caffeine was also nearly identical to that for the 25-mg/kg dose. However, the greater specific activity of the 5-mg/kg dose allowed a few additional trace peaks to be observed. None of these corresponded in R_f to 1-methylxanthine. The additional uric acid chromatographic peaks observed were mainly unidentifiable hydrophobic compounds, and the additional non-acid chromatographic peaks were non-xanthine hydrophilic compounds, presumably resulting from further catabolic reaction of the caffeine molecule. In all of the organs examined (Tables 1, 2 and 3), it was apparent that only a small percentage of the administered radioactivity could be found as "acids," in contrast to the results obtained with urine, where these "acids" often make up 50 per cent of the nonvolatile radioactivity.[6]

Rather than repeat the entire quantitative analysis of the concentration of caffeine and its metabolites within specific organs for animals which had received caffeine in their drinking water since weaning, the distribution of caffeine-derived radioactivity in these animals 24 hr after the administration of 10 mg/kg of 1-methyl[^{14}C]caffeine was determined by whole body radioautography.[13] Frozen carcasses were sectioned to yield whole body sections and these were placed in contact with X-ray film for 3·5 months. The developed radioautograms were compared to similar radioautograms prepared from animals which had received 10 mg/kg of [^{14}C]caffeine 24 hr prior to sacrifice but which had not previously been exposed to caffeine.*

The results obtained by radioautography with animals receiving a chronic oral dose of 4, 15 or 30 mg/kg/day of caffeine as compared to those receiving caffeine for the first time indicated no significant difference in the distribution of caffeine-derived ^{14}C-radioactivity. Some evidence was seen for a generally longer retention of radioactivity

* R. Liss, unpublished observations.

in the chronically treated animals. However, the relative distribution and tissue retention between organs as determined in the 24-hr sections were identical in both experiments. Specifically, no difference in localization as determined by the tissue/blood image density ratio was observed. The chronic administration of caffeine apparently did not result in an altered concentration of caffeine-derived radioactivity in any organ or tissue of the mouse.

Although the major metabolites of caffeine had been identified and the disposition of the administered caffeine had been reasonably described, it was important to us to determine whether an amount of caffeine even less than 0·01 per cent of the administered radioactivity was metabolized to a methylxanthine nucleotide such that incorporation into nucleic acid could occur. A separate specific investigation of liver homogenate was thus conducted to determine the presence of radioactivity in nucleic acid and nucleotides after the administration of [^{3}H,^{14}C]caffeine, using gradient elution column chromatographic techniques as described in Methods.

Figure 1 illustrates the elution profile of ^{14}C-radioactivity from a column to which had been applied a sample of liver homogenate acid extract obtained from a mouse 3 hr after the administration of 25 mg/kg of [^{3}H,^{14}C]caffeine. Samples obtained 1 and 8 hr after dosing with caffeine were also prepared. Peaks with identical elution characteristics were observed with each of these samples. In the case of the 1-hr sample, the

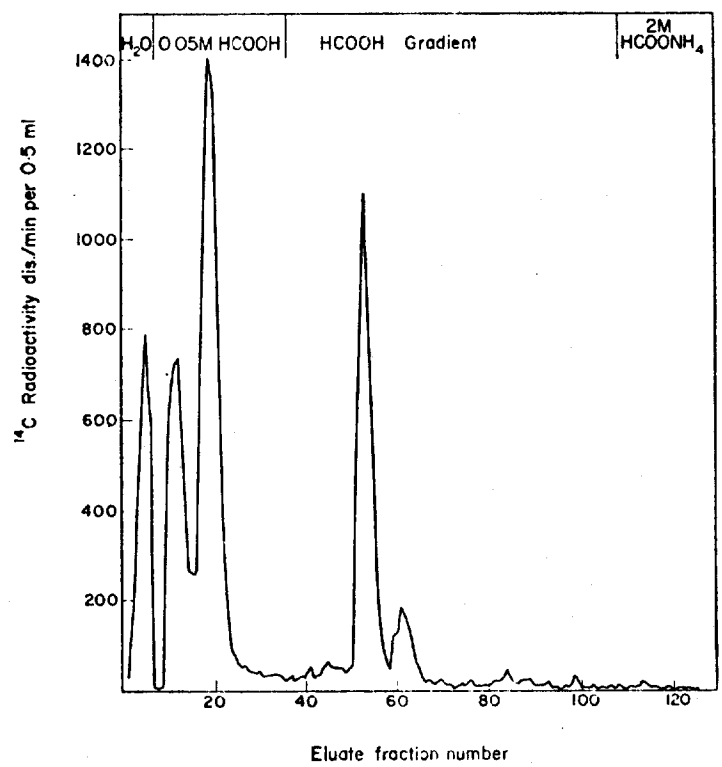

FIG. 1. Elution pattern from an AG-1-formate column to which an acid extract of liver had been applied. The liver was obtained from three mice which had been given 25 mg/kg of [^{3}H,^{14}C]caffeine, p.o., 3 hr previously. The composition of eluant is indicated; 3·0-ml fractions were collected.

second and third peaks were twice the size of those seen in Fig. 1, however, and with the 8-hr sample all peaks were smaller, as would be expected, based on the low level of radioactivity present in the liver 8 hr after dosage. Tritium radioactivity eluted from the column directly paralleled ^{14}C-radioactivity except that the peak observed eluting at tube 61 contained no tritium, and with the 8-hr sample, two small peaks containing tritium but no ^{14}C were eluted in the vicinity of tube 80. Only the 3-hr 25 mg/kg sample resulted in radioactivity being eluted by the 2·0 M ammonium formate final eluant.

Nucleotide di- and triphosphates would be expected to be eluted from the columns between tubes 110 and 126. As can be seen from Fig. 1, only an insignificant amount of radioactivity was found in this area. The peak at tube 114 was only twice background and was not present at all in the 1-hr or 8-hr samples. Nucleotides might appear anywhere in the formic acid gradient. A considerable portion of the radioactive material present in the liver homogenates (25 per cent) does elute here. It is also possible, however, that the radioactivity in this area might be due to other acids such as methyluric acids. Uric acid itself, however, would be expected to elute earlier from the column.

To determine if the large peaks eluted by the formic acid gradient represented nucleotides or uric acids, a sample of the acid fraction of urine obtained 3 hr after the administration of a 5-mg/kg dose of [^{3}H,^{14}C]caffeine was placed on an AG-1-formate column and chromatographed in the same fashion as the liver homogenate. Most of the radioactivity eluted in the water and formic acid washes, as it did with the liver preparation. However, two additional peaks in the formic acid gradient, representing 26 and 1·4 per cent of the ^{14}C-radioactivity were observed. The peaks corresponded closely to the two "nucleotide" peaks observed with the liver acid extract. The appearance of compounds resulting in such peaks in the urine indicated that the two peaks from liver are not associated with nucleotide material, since it is highly unlikely for nucleotides to exist in any abundance in the urine. It is most probable that these peaks are, in fact, methylated uric acids. When the total acid fraction obtained from the various liver homogenates was compared to the amount of material in the AG-1-formate eluate in tubes 44–69, a constant ratio of 1·4–1·7 was observed, further strengthening the conclusion that these two peaks result from uric acids.

To investigate further the possibility that methylxanthine nucleotides by coincidence are eluted concomitantly with methyluric acids, the material in tubes 44–69 was chromatographed on a cellulose thin-layer system with 95% ethanol–1 M ammonium acetate, pH 7·5, 35:70, both before and after treatment with alkaline phosphatase. In this system, inosine migrated with an R_f value of 0·65, inosinic acid with an R_f of 0·47, and uric acid with an R_f of 0·30. The thin-layer plate was scanned for radioactivity with the Packard strip scanner. The plate was then divided into small areas which were scraped into scintillation vials, eluted with water, and counted in the scintillation spectrometer in TEN scintillator solution. No significant differences were seen between the developed chromatograms with and without alkaline phosphatase treatment.

Finally, the low level of radioactive material existing between tubes 73 and 93 was combined and chromatographed in a 5 M ammonium acetate (pH 9·0)–saturated sodium tetraborate–95% ethanol–0·5 M versene (40:160:360:1) thin-layer system. Once again, no difference was observed in the distribution of radioactivity between the control and the alkaline phosphatase-treated eluate. The absence of radioactivity at the origin of the control sample where the nucleotide would remain also led to the

conclusion that the material eluting in tubes 73–93 was probably not a methylxanthine nucleotide.

Significant radioactivity was incorporated into the DNA and RNA fractions of liver. Three hr after administration of 25 mg/kg of [^{3}H,^{14}C]caffeine, p.o., 0·02 per cent of the ^{14}C and 0·01 per cent of the ^{3}H was associated with DNA in this organ, and the liver RNA contained 0·04 and 0·03 per cent of the respective administered radioactivity. After 8 hr, the liver nucleic acid fractions still contained 0·01 per cent of the administered radioactivity in DNA, and 0·02 per cent ^{14}C and 0·04 per cent ^{3}H in RNA. This radioactivity represented less than one-fifth of the radioactivity associated with the acid-insoluble fraction at these times and is probably the result of random incorporation of labeled compounds resulting from the extensive metabolic decomposition of caffeine. This view was supported by the finding that, whereas the distribution of radioactivity (both ^{14}C and ^{3}H) between RNA and DNA remained constant from 3 to 8 hr after dosing, the ratio of ^{3}H/^{14}C increased over 2-fold in this same time period in both nucleic acid fractions. No attempt was made to determine which monomeric elements of the nucleic acid were radioactive.

DISCUSSION

The data of Tables 1, 2 and 3 were recalculated to compare the concentration of caffeine in each organ to the water content of that organ (Fig. 2). In the interests of clarity, the results for all organs investigated are not presented graphically. Heart and muscle, for instance, gave results almost identical to those for lung. At the 1-hr period following dosage, the milligrams of caffeine per kilogram of tissue water in the lung, heart, muscle, testes and liver was 17·1, 17·4, 17·5, 17·6 and 17·8 respectively. Such results tend to support the finding in the dog and man that caffeine distributes with body water.[2,3] The more detailed analysis presented in Fig. 2, however, indicates that at the earlier and later time periods the value of caffeine/tissue water did differ from one organ to another. The uptake and excretion of caffeine from these organs did vary and a direct correspondence between caffeine concentration and tissue water resulted only during a short plateau period.

Specifically, it would seem that a barrier to the accumulation of caffeine in the brain and testes existed, while the liver, spleen and perhaps kidney actively removed caffeine from the plasma. This is in agreement with published data on the distribution of total radioactivity in the liver, brain and testes of mice after the injection of tritiated caffeine.[16] The kidney may maintain a higher concentration of caffeine because of the presence of compound in the tubules. However, the low value for caffeine concentration in the plasma, apparent after both the 5- and 25-mg/kg doses, indicates that in the body of the mouse caffeine did not have an opportunity to establish a true equilibrium concentration.

Caffeine had a half-life of less than 3 hr in all organs investigated and apparently the rapid metabolism and excretion of caffeine metabolites were responsible for this.

After a 5-mg/kg oral dose of caffeine, a shorter tissue half-life for caffeine was observed than after a 25-mg/kg oral dose. This decreased half-life of caffeine was independent of the half-life for total radioactivity administered, which was the same after each dose. Specifically, a plasma half-life of 2·5 hr for total ^{14}C radioactivity was observed after both oral doses. The half-life of caffeine, however, was calculated

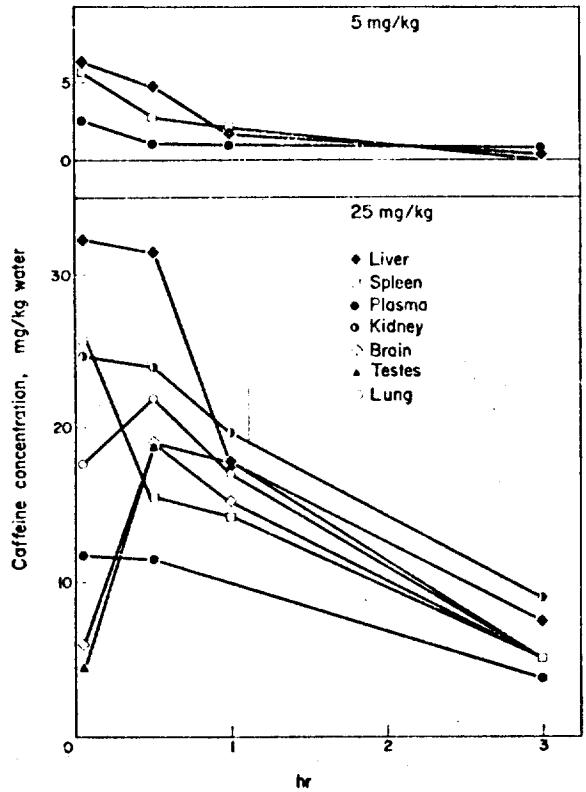

FIG. 2. Caffeine in organs of the mouse expressed as the concentration in tissue water. Caffeine was determined by multiple chromatography of tissue homogenates obtained from groups of three mice sacrificed at various times after the administration of 5 or 25 mg/kg of [^{3}H,^{14}C]caffeine, p.o. Tissue water was calculated based on organ wet weights and values obtained from the literature.[14,15]

to be 1·7 and 0·6 hr after 25 and 5 mg/kg of caffeine respectively. Such findings illustrate the difficulty in drawing conclusions concerning the dynamics of tissue distribution solely from total radioactivity data.[17] In this case the smaller dose was more extensively metabolized, but these products of metabolism were not, in general, excreted more rapidly.

One class of compounds was excreted rapidly. Very little methyluric acid could be demonstrated in the tissues of the mouse, although 1-methyluric acid and 1,3-dimethyluric acid have been shown to be major urinary metabolites in this species[6] as well as in man.[18] The uric acid metabolites must readily pass into the blood and be removed by the kidney. Thus the high concentration of uric acids in the urine gave no true indication of the exposure of tissue to these particular caffeine metabolites.

In contrast, the 1,7-dimethylxanthine which is found in the urine does not reveal that 1,7-dimethylxanthine is the major caffeine metabolite to which the mouse organs were exposed. Other dimethylxanthines absent from the urine were also absent in the tissues, although low levels of theobromine (as well as 3-methylxanthine and 7-methylxanthine) could have been missed due to the difficulties of measuring trace quantities of tritium-labeled metabolites in tissue by our methods. No 1-methylxanthine, a major

urinary metabolite in man[18] but not in the mouse,[6] was detectable. Neither could the presence of methylated nucleotides resulting from caffeine be shown. The presence of such compounds would have had implications for the relationship of caffeine and nucleic acid function.

The only metabolite of caffeine which had been previously reported in a mammalian tissue is 1,7-dimethylxanthine. The administration of 500 mg caffeine to human subjects by Warren[19] resulted in the appearance of 1,7-dimethylxanthine in the plasma and erythrocytes within 3 hr. Theophylline and theobromine were not observed. 1,7-Dimethylxanthine is the major metabolite of caffeine present in the tissues of the male CD-1 mouse and may prove to have a like role in other mammalian species.

TABLE 4. CONCENTRATION OF CAFFEINE AND 1,7-DIMETHYLXANTHINE IN THE ORGANS OF THE MOUSE OVER 24 hr

	C × t (mg hr/kg)			
	5 mg/kg dose		25 mg/kg dose	
Organ	Caffeine	1,7-CH_3-xanthine	Caffeine	1,7-CH_3-xanthine
Brain	3·5	<2	38	10
Heart	2·4	4·5	50	<10
Kidneys	5·9	9·9	55	30
Liver	5·4	2·8	45	24
Lungs	3·6	3·3	39	10
Spleen	2·6	<2	35	<10
Testes	5·0	<2	46	19

Table 4 presents a comparison of the exposure of organs of the mouse to caffeine and its major metabolite. The data in this table were obtained by actually measuring the areas under the concentration vs. time curves obtained for caffeine and 1,7-dimethylxanthine. These caffeine values (Table 4) should be compared to the theoretical C × t values of 120 and 600 mg hr/kg for a 5- and 25-mg/kg administered dose in the absence of excretion or metabolism.

Even considering the C × t exposure of organs over the 8 hr during which caffeine concentration was measurable or could be directly extrapolated, the exposure is less than one-fourth the theoretical value, assuming uniform equal distribution in body water. With two exceptions, the organs experienced an even lower exposure to 1,7-dimethylxanthine. The between organ variation in 1,7-dimethylxanthine exposure was much greater than that for caffeine exposure, indicating a different rate of formation or absorption between organs for this compound. No evidence for accumulation of 1,7-dimethylxanthine in any organ was apparent. The lack of organ-specific accumulation of any other ^{14}C metabolite was demonstrated by the radioautographic experiment. This experiment also revealed that prior chronic administration of caffeine did not significantly alter the level of residual ^{14}C-radioactivity in the mouse 24 hr after administration of 10 mg/kg of [^{14}C]caffeine.

The ability of caffeine to stimulate the activity of hepatic drug-metabolizing enzymes in the rat has been reported.[20] In the mouse, active microsomes from liver and the cytosol-microsome fraction from kidney failed to demethylate caffeine or theobromine

(unpublished observation). Mazel and Henderson[21] have observed less than a 0·4 per cent demethylation of caffeine, theobromine or theophylline, a level which would have been readily detected in our experiments. These low conversions seem inconsistent with the rapid metabolism of caffeine in the mouse. Likewise, we and others[22] have demonstrated the inability of bovine milk xanthine oxidase to oxidize caffeine or theophylline to the corresponding uric acids (1-methylxanthine is oxidized), although methyluric acids are a major metabolite.

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GENETICAL EFFECTS OF CAFFEINE IN MICE

By

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Introduction

The importance of testing for possible mutagenic effects of substances that are used as preservatives or are constituents of human food has been stressed by HALDANE (1956). Tests of this kind are best made on mammals where the results may be more suitable for analogy with human populations.

The present communication concerns tests of caffeine for its ability to induce translocations in mouse chromosomes. Caffeine (trimethylxanthine) was selected for test since it is an active constituent of tea, coffee, and cocoa and has been shown to have mutagenic properties in a variety of other organisms (KIHLMAN and LEVAN 1949; NOVICK and SZILARD 1951; GLASS and NOVICK 1959; ANDREW 1959). The results indicate that caffeine is only weakly mutagenic in mice, for only two possible translocations were detected. An effect upon the fertility of the treated animals was, however, observed.

Methods and materials

GOLDSTEIN (personal communication) has shown that C57/DBA mice will accept caffeine in their drinking water up to a concentration of 3 gms/litre and will maintain weight apparently indefinitely. He suggested that this dose would be the most suitable for genetic experiments. Preliminary tests with the strain of mice used in the present experiment were in agreement with this suggestion. 3 gms. caffeine per litre of drinking water was the highest dose that could be administered without noticeably affecting the health of the animals.

Fourteen inbred (JU) males were therefore given 0.3% solutions of caffeine as their drinking water and this was renewed each week. Treatment was continued for three months to ensure that the earliest spermatogonia were subjected to the caffeine administration for some time. At the end of this period each male was placed with three virgin females from a randombred stock (Q) and left for six weeks. The females were examined each morning for vaginal plugs and those which had copulated were removed and replaced by new virgin females.

The mated females were placed in separate cages and allowed to go to term when the litter-sizes were scored. All the offspring were kept until three weeks old, at which time they were examined for morphological abnormalities, and the daughters then discarded. The sons were kept until at least six weeks old when they were tested for the possession of a translocation. Preliminary screening was done by fertility tests. Each male was placed with two virgin Q stock females and the litter sizes were scored at birth. Those males which had an average litter-size of less than eight among the litters of both females were suspected of being heterozygous for a translocation. Such males were retested and a cytological examination of the testes was carried out if the litter size remained low. Two cytological techniques were employed: firstly, preparations from one testis were made by the method of WELSHONS et al. (1962) and secondly, if further analysis was required, preparations from the other testis were made by the method of SLIZYNSKI (AUERBACH and SLIZYNSKI 1956). The existence of nineteen instead of twenty separate elements seen in at least eight metaphase plates of the first meiotic division was the criterion used to indicate the presence of a translocation.

Results

The Table illustrates the fertility of the caffeine-treated males mated to virgin *Q* stock females, and also the data on the first litters of the *Q* stock of the same generation which are here used as the control. The litter size of the treated series was lower than that of the control and the difference was significant at the 0.1% level of probability ($t_{(238)} = 5.56$). Of more value, however, was the fact that the litter size tended to increase with time after treatment until the sixth week, at which time it was close to the control level. A regression of individual litter size on day conceived after cessation of treatment of the fathers indicated that this trend was significant at the 2% probability level ($t_{(137)} = 2.38$). Although data on the incidence of zero litters in the control were not available, the number of sterile matings in the treated series seemed rather excessive. This predominantly occurred among females mated in the first three weeks. A χ^2 analysis for a trend was not significant ($\chi^2_{(3)} = 4.9$) but there was a significant difference between the incidence of zero litters in the first three weeks and thereafter ($\chi^2_{(1)} = 4.35$).

Table. *The fertility of males treated for three months with a 0,3 % solution of caffeine as their drinking water*

Days after end of treatment	No. of fertile females	No. of offspring	Av. litter size of fertile females	No. sterile females	% sterile matings
1—7	34	232	6.82 ± 0.58	10	22.72
8—14	32	238	7.44 ± 0.52	5	13.51
15—21	21	142	6.16 ± 0.83	10	32.15
22—28	23	199	8.65 ± 0.54	3	11.54
29—35	20	150	7.50 ± 0.67	1	4.76
36—60	13	121	9.30 ± 1.04	2	1.33
Total	143	1082	7.57 ± 0.27	31	17.82
Control	97	949	9.78 ± 0.27	—	—

Breeding tests were carried out on 201 F_1 sons, all conceived in the first two weeks after the end of treatment of the fathers. Of these, only 13 males produced litters with an average size lower than eight, and only one of the thirteen had an average of less than five offspring per litter. Cytological analyses of all 13 animals were carried out and 11 were considered to be quite normal, i.e. no indication of a translocation was observed in over 40 cells scored. Two males, however, showed cytologically abnormal pictures, but in neither case was there an association of four chromosomes, or nineteen instead of twenty separate elements. The somewhat unusual chromosome pictures in these cases may be due to some other types of rearrangement, and Dr. SLIZYNSKI of the department is undertaking further analysis of both males and their offspring.

No morphologically abnormal animals were observed in either the F_1 or F_2 generations.

Discussion

201 sons of caffeine treated males were tested for the possession of translocations. All were conceived within two weeks of the end of treatment of the fathers, and thus were derived from germ cells that had received the maximum amount of treatment, i.e., both as pre- and post-meiotic germ cell types. It was found that not one of the 201 F_1 animals possessed a translocation of the type searched for, i.e. one reducing fertility by about 50% and cytologically detectable as an association of four chromosomes at meiosis. Two animals had slightly reduced fertility (average litter sizes 6.7 and 7.3) and appeared to have some chromosomal abnormalities, the nature of which has not yet been determined. Conceivably

many more rearrangements of this type might have occurred, but remained undetected since it is only by chance that the two abnormal animals were picked up, many of their individual litters being well above the set standard. If this were the case, however, it would be surprising if translocations that markedly affect fertility were not also produced, unless it were postulated that caffeine tends to induce rearrangements of a particular type. While a tendency for certain chromosome regions to be affected more readily than others by certain chemical mutagens is known in *Drosophila* (FAHMY and FAHMY 1956, BELITZ 1957) it is most unlikely that this could account for the complete lack of translocations that produce semi-sterility in the present experiment. Since the tested animals represent treated post-meiotic as well as pre-meiotic germ cells, germinal selection cannot account for the absence of translocations. These data therefore indicate that a 0.3% caffeine solution, administered orally to male mice, is at most only weakly mutagenic.

Contrary to the essentially negative results of the translocation tests, a true reduction in fertility of the treated animals was detected. The reduced fertility was deduced from two observations: litter size was lower than in the controls, and it increased with time after cessation of treatment. The first observation in itself is not sufficient proof that caffeine treatment lowered fertility. For two reasons the data from the *Q* stock do not provide an accurate control: First, the males employed in the treated series were from a different and inbred stock. Second, the control data were collected from one generation of 30 matings (2 ♀♀: 1 ♂ per mating) producing litters at approximately the same time, while in the treated series 14 males were mated frequently to numerous females to produce litters over a six-week period. The reduced litter size might therefore be due to the fact that the inbred males used in the experiment either had a lower fertility than *Q* stock males, or that the frequent mating tended to make them less fertile. Yet these factors are hardly likely to account for a reduction of approximately two young per litter, for FALCONER (1960) has estimated that the variation between litters attributable to the male is small (of the order of 10%) compared with the influence of the female parent and the litter itself. Furthermore, the fact that the litter size tended to rise to the control level invalidates the idea that frequent mating is the cause of the lower fertility.

More valid evidence for a caffeine-effect on male fertility is provided by the increase in fertility with time after treatment. This is seen as an increase in litter size of fertile females and as a decreasing incidence of sterile matings. If zero litters were included in the calculations of litter size, the reduction in fertility and the trend towards full fertility would be much more obvious.

A rise in fertility with time after treatment might simply be due to the fact that the females producing the late litters were on the average older than those producing the early ones. Since, however, FALCONER (unpublished) has shown that the number of young in first litters does not depend on the age of the dam, this explanation appears unlikely. A much more plausible explanation of the observed change in fertility is the different type of treatment received by spermatozoa used for the early and late litters: while the first had been exposed to caffeine in all stages of spermatogenesis, the latter had been treated only as immature germ cells. If OAKBERG's (1956) timing of spermatogenesis in the mouse is valid also

for caffeine-treated males, spermatozoa used 5—6 weeks after treatment—when litter size had returned to the control level—would have been exposed only as spermatogonia, from which dominant lethals cannot usually be recovered after any type of treatment.

A genetic base of infertility agrees with the results obtained with caffeine in a variety of other organisms. Thus caffeine induced back-mutation to phage-resistance in *Escherichia coli* (NOVICK and SZILARD 1951; GLASS and NOVICK 1959), it increased the frequency of biochemical mutants in *Ophiostoma multiannulatum* (FRIES and KIHLMAN 1948), and it produced chromosome breakage in *Vicia* root-tip cells (KIHLMAN and LEVAN 1949). In addition caffeine has been found to be weakly mutagenic in *Drosophila* where an indication of greater sensitivity of post-meiotic as compared with pre-meiotic germ cells was noted (ANDREW 1959). On the other hand LYON *et al.* (1962) obtained no evidence for genetical effects of caffeine on male germ cells of the mouse. No visible mutations occurred in a large scale test of treated spermatogonia. More important for the present discussion is the negative result of tests for dominant lethals, carried out on post-meiotically treated spermatozoa. Different doses were used in these tests, most of them lower than that used in the present experiment. Since at higher doses the frequency of dead implants showed a tendency to increase, the discrepancy between these findings and the results reported here may be more apparent than real.

A more weighty argument against attributing the decreased fertility in the present experiment to dominant lethals arises from the fact that in this same experiment translocations—at least of the usual type—were not produced. Triethylenemelamine (TEM), which does reduce fertility by inducing dominant lethals, produced 4.5% translocations in experiments in which fertility was depressed to about the same extent as in the caffeine experiment (CATTANACH 1959). Without *ad hoc* assumptions, such as the production of breaks without rejoining ability, it is indeed difficult to see how a chemical that produces dominant lethality through chromosome breakage should fail to produce at least some viable rearrangements.

It may therefore be useful to look for alternative explanations of the caffeine-induced reduction in fertility. Possible causes are suggested by its demonstrated effects on the nuclei of other species. In bacteria caffeine increases the time-lag between mitotic cell divisions (WITKIN 1958; LIEB 1961). If it had a similar effect on mouse meiosis, the reduction in litter size could be the consequence of oligospermia. STIEVE's (1928) finding that testicular damage with agglutinated sperm and reduced germinal epithelium may be induced in rabbits by caffeine-treatment provides support for this hypothesis. In *Vicia* caffeine causes chromosome stickiness and anaphase lagging (KIHLMAN and LEVAN 1949). If this occurred in the meiotic divisions of the treated mice, or in the early cleavage divisions of eggs fertilised by treated spermatozoa, it might lead to non-disjunction followed by lethal aneuploidy. An experiment to test if caffeine increases the frequency of non-disjunction of the sex-chromosomes of the mouse is now in progress.

The reduction of fertility may thus be attributed to any of several mechanisms and the present data do not distinguish between them. An attempt to do so by a more thorough analysis has been started.

Summary

Caffeine administered orally in the drinking water as a 0.3% solution is not a very effective mutagen in the mouse. It may be mutagenic in its effects on male fertility, but it is ineffective in producing translocations that cause semi-sterility.

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EFFECT OF CAFFEINE VERSUS PLACEBO SUPPLEMENTATION ON BLOOD-GLUCOSE CONCENTRATION

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Summary In twenty-two presumably healthy third-year dental students blood-glucose levels er oral ingestion of 250 mg. caffeine were significantly her than the blood-glucose levels in twenty-one dents ingesting a placebo.

Introduction

THERE is increasing recognition of the diagnostic and sible prognostic significance of carbohydrate meta-sm in seemingly non-diabetic syndromes such as ltiple sclerosis (Cerkez and Chandler 1962), gout (Berkowitz 1966), carcinomatosis (Lisker et al. 1966), mental illness (Salzer 1966), periodontal disease (Cheraskin 1960), arteriosclerosis (Kingsbury 1966), and ischæmic heart-disease (Gelfand and Fabrykant 1965).

Blood-glucose concentration, the most commonly employed barometer of carbohydrate metabolism, is the resultant of a host of hormonal and dietary factors. Surprisingly, despite the large consumption of caffeine-containing substances, little attention has been directed to the possible effects of caffeine on blood-glucose content. Deakins (1939) observed from four glucose-tolerance tests performed on himself at different times with caffeine and coffee, that large doses of caffeine depress the peak of the curve and delay the return of blood-glucose to baseline values. He concluded, from these limited studies, that caffeine per se did not elevate blood-glucose appreciably. Bellet et al. (1965) studied the plasma-glucose values during a three-hour period following ingestion of coffee, water, caffeine sodium benzoate, and sodium benzoate. They reported that there were no significant changes in blood-glucose. Hankiewicz (1960) investigated the blood-sugar in 228 patients, including 176 diabetics, before and after administration of caffeine by injection and by mouth as caffeine sodium benzoate and coffee. He concluded that caffeine depressed blood-sugar and that the effect was related to the dose of the drug and to the blood-glucose concentration. Iancu et al. (1957), however, observed in normal and cortically inhibited children that caffeine produces varying degrees of hyperglycæmia. Jankelson et al. (1967) found, in nine volunteers with maturity-onset diabetes, blood-glucose levels (after intravenous glucose, 0·5 g. per kg. body-weight) significantly higher following two cups of "instant" coffee than when water had been drunk.

Because of the ubiquity of caffeine consumption, the diagnostic and prognostic potential of blood-glucose, and the varied published results, this study was designed to reinvestigate the relation of caffeine to blood-glucose in a sample of young and presumably healthy subjects.

Materials and Methods

Forty-three junior dental students presented themselves fasting at 10 A.M. A venous blood sample was obtained and blood-glucose measured (Nelson 1944, Somogyi 1945). Immediately after the blood was drawn, the group was randomly divided. Twenty-two subjects were asked to swallow a capsule containing 250 mg. caffeine (*U.S.P.*) with 5 oz. of water; the remaining twenty-one took an indistinguishable placebo capsule containing lactose. Blood samples were again obtained half an hour and one and two hours later.

Results and Discussion

Table I shows the means and standard deviations for age of the volunteers and the blood-glucose values. There is no statistically significant difference of the means of the ages of the two groups (utilising the Student

TABLE I—STATISTICAL SIGNIFICANCE OF CAFFEINE-VERSUS-PLACEBO SUPPLEMENTATION ON THE BLOOD-GLUCOSE CONCENTRATION

—	Placebo group	Caffeine group	Significance of differences of means
Age (yr.)	24·9 ± 3·5	26·8 ± 5·5	$P > 0·100$
Blood-glucose:			
Fasting	86·1 ± 6·1	87·1 ± 6·9	$P > 0·500$
½ hr.	86·1 ± 6·3	90·1 ± 6·4	$P < 0·050$*
1 hr.	86·3 ± 8·1	90·7 ± 6·2	$P > 0·050$
2 hr.	85·9 ± 7·5	93·0 ± 6·8	$P < 0·005$*

*Statistically significant.

TABLE II—STATISTICAL SIGNIFICANCE OF THE DIFFERENCES OF THE MEANS

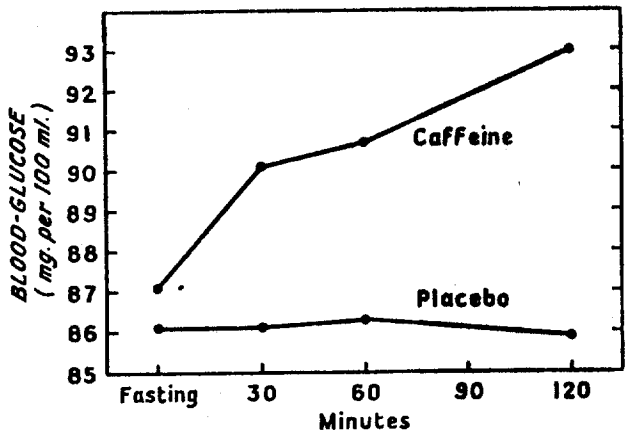
Group	Fasting blood-glucose versus:		
	½ hr.	1 hr.	2 hr.
Placebo ..	$P > 0.5000$	$P > 0.5000$	$P > 0.5000$
Caffeine ..	$P > 0.1000$	$P > 0.0500$	$P < 0.0005$*

*Statistically significant.

t test). With regard to blood-glucose, the half-hour and the two-hour levels are statistically significantly different ($P < 0.050$ and < 0.005), and the one-hour level borders on statistical significance at the 5% confidence level. In other words, the mean blood-glucose at a half and two hours after caffeine ingestion is significantly higher than for the placebo group.

The remaining question is whether there were significant changes *within* the groups with the passage of time. Table II shows that, in the placebo group, there are no significant variations when the fasting mean blood-glucose is compared with the findings at a half, one, and two hours. In contrast, it is clear that the blood-glucose two hours after caffeine supplementation is significantly higher than that under fasting conditions. (A comparison of the fasting and one-hour scores borders on significance at the 5% confidence level.)

As the figure shows the mean blood-glucose values in time following the placebo supplement are strikingly steady. Thus the means are consistently close to 86 mg. per 100 ml. and the standard deviations regularly cluster about 6–7 mg. per 100 ml. The importance of this homœostatic picture is underlined (table II) by the lack of a significant difference between the temporal points. In



Effect of caffeine-versus-placebo supplementation on blood-glucose studied during a two-hour period.

contrast, the mean blood-glucose scores in the caffeine-supplemented group rise slowly and significantly with time (table II and figure).

Although the blood-glucose response to caffeine reported here is not of great magnitude, it may produce physiological reverberations. Luft et al. (1966) observed that a reduction in blood-glucose of less than 10 mg. per 100 ml. was accompanied by a significant elevation in plasma-human-growth-hormone and a significant increase in the urinary output of adrenaline.

Since coffee is consumed in large quantities throughout the lives of many, including those who may have a predisposition to maturity-onset diabetes, its effect on glucose homœostasis should be carefully explored.

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THE EFFECTS OF CAFFEINE AND THEOBROMINE UPON THE FORMATION AND EXCRETION OF URIC ACID[1]

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Both caffeine and theobromine are consumed, in a large measure, in our common beverages (coffee, tea, cocoa, chocolate, etc.). During the past eleven years the average per capita consumption of coffee in the United States has been 11.6 pounds (a minimum of 8.9 pounds in 1913 and a maximum of 12.8 pounds in 1920,—reports of C. E. Bickford & Company, coffee brokers, San Francisco, California) and approximately one pound of tea. Assuming that the average caffeine content of coffee be 1.5 per cent and that of tea 2.5 per cent, this would give an annual per capita consumption of approximately 90 grams, or 225 milligrams of caffeine per day. This per capita consumption is misleading because many children and non-users of tea and coffee have been included in the figures used. Moreover only two sources of caffeine and other methylated xanthine compounds (theobromine, theophylline) have been considered. In addition to their ingestion in beverages both caffeine and theobromine are used extensively in therapeutics.

There has been considerable dispute as to the effect of these substances upon the formation and excretion of uric acid. The work thus far has been, for the most part, limited to a study of urinary excretion. The probable association of residual uric acid with such clinical manifestations as gout, arthritis and urinary calculi emphasizes the importance of a simultaneous study of its concentration in the blood and the urinary excretion.[4]

It has been suggested that the effects of caffeine and theobromine might

[1] Aided by a grant from the Research Board of the University of California.

[2] Part of the work presented in this paper is taken from a thesis submitted by Alfred A. de Lorimier in partial fulfilment of the requirements for the degree of Master of Science in the Graduate School of the University of California.

[3] A preliminary notice of this work appeared in the abstracts of the Journ. Pharm. Exper. Therap., 1925, xxv, 167.

[4] Even a study of this nature neglects the consideration of the excretion of uric acid in the sweat and by way of the intestinal tract.

vary after their prolonged ingestion. Therefore the experiments reported in this paper have been extended over a long period as outlined below. Since it is a well-known fact that man, in contrast to experimental animals, possesses (at the most) a low power of destruction of uric acid, we have used human subjects for this work. These were healthy men picked from a list of fifty or more volunteers amongst the inmates of the prison at San Quentin. These men were placed on the following "low purine" diet.

ARTICLES	DAILY ALLOWANCE
Milk (raw)	500 cc.
Cheese	50 grams
Butter	60 grams
Jelly	30 grams
Raisins (seeded)	25 grams
Tomatoes (canned)	40 grams
Shredded wheat	60 grams approximately (2 biscuits)
Graham crackers	100 grams
Bread (Graham)	200 grams approximately
Potatoes	200 grams
Sugar	50 grams
Hard candy (pure cane sugar)	30 grams
Postum	25 grams (powdered substance)
Sodium chloride	4-5 grams (as desired)
Rice	75 grams (dry weight—three times per week

Although the individual apportionments of these substances varied to a slight extent they were kept constant for each subject throughout the experimental period.

PLAN OF EXPERIMENTS. *Part A.* During the first set of experiments observations were made upon four subjects for a period of ten weeks.

1. A preliminary period of 5 days was allowed to accustom the men to the new diet and thereby to judge the quantities of each food which were to be maintained constant throughout the ensuing periods.
2. Control period, 7 days (low purine diet).
3. Caffeine period, 10 days. (Same diet as above with the addition of oral doses of caffeine, varying from 0.25 to 1.0 gram per day.)
4. Control period, 11 days. (Low purine diet.)
5. High protein, high purine diet. 3 days. (Liver ingestion.)[5]
6. Control period, 7 days. (Low purine diet.)

[5] Followed by four days on a generous meat diet, during which time no data were obtained.

7. Theobromine period, 7 days. (Same diet as above with the addition of oral doses of theobromine, varying from 0.5 to 1.75 grams per day.)

8. Control period, 8 days. (Low purine diet.)

9. Theobromine period, 3 days. (Regular low purine diet to which the more highly soluble theobromine salts (acetate, salicylate) were given orally in doses from 1.5 to 2.0 grams per day.)[6]

Part B. The diet was essentially that described under part A: three subjects were used, only one of whom (J. W.) went through the part A experiments. In this second set of experiments, which lasted about two weeks, uric determinations were made at frequent intervals during the day.

1. Control period, 7 days. (Low purine diet.)

2. Control day, with frequent blood and urinary determinations.

3. Caffeine day. (Same diet as above with the addition of 750 mgm. of caffeine at breakfast and the same routine of analyses.)

4. Control period, 3 days. (Low purine diet as above.)

5. Control day with frequent blood and urine determinations.

6. Theobromine and caffeine day. (Same diet as above with the addition at breakfast of 1950 mgm. of theobromine sodium salicylate for subjects F. G. and J. W., and 1000 mgm. of caffeine for subject C. M. The regular routine of analyses.)

7. "Follow-up" day. Urinary analyses only. (Regular diet.)

METHODS. *Part A.* The blood samples were taken before breakfast twice a week by ordinary venepuncture, using lithium oxalate as the anticoagulant. Within ten minutes of the time the sample was obtained the blood was deproteinized by the Folin-Wu (1) method. The uric acid determinations were made on these blood filtrates within six hours, by Benedict's (2) method.

Urine samples were collected over 24-hour periods. An excess of chloroform was kept in the collecting bottles and storage containers to prevent bacterial decomposition. Uric acid was determined by the Benedict and Franke (3) method within four hours after the 24-hour sample was complete.

Part B. The analytical methods and the general procedure were the same as in A except that the blood and urine samples were obtained in two and three hour periods, as outlined in charts 9, 10, 11.

DISCUSSION. Before discussing the actual experimental results presented in this paper let us consider briefly certain variables which might modify the physiological action of these drugs. In the first place caffeine and theobromine were given orally in our experiments and it is therefore impossible to estimate either the rate or the total amount absorbed. Ab-

[6] These highly soluble theobromine compounds (sodium theobromine acetate and salicylate) were given in order to check up the absorption of the less soluble theobromine base. No difference in action was observed.

sorption is dependent not only upon the cellular activity of the mucosae, but also upon such factors as the bulk of the food residues, bacterial activity, peristaltic rate, etc., all of which are affected by energy output (work, rest, temperature). Secondly, the same factors which influence absorption of a drug also play a part in the rate of excretion (by way of the urine, perspiration or feces). A third important factor is that of tolerance. In one instance we may be dealing with an individual who has acquired a tolerance by long use of caffeine or theobromine beverages and who would therefore give a less intense or a more prolonged reaction than the individual who had received the drug for the first time. Finally, there is the probability that varying amounts of uric acid undergo decomposition in the blood and tissues.[7] The numerous environmental factors mentioned above might also play a part here.

The environmental and energy factors were perhaps the most constant in our subject, E.B., who was a librarian. A glance at chart 1 shows that the first dose of caffeine, 250 mgm., caused a marked initial increase in the excretion of uric acid (approximately 35 per cent above the average, or 23 per cent more than the highest value of the 10 day control period). This immediate rise was followed by a gradual decrease until a second stimulating effect was obtained by a dose of 500 mgm., which in turn was followed by a similar decrease and a third rise when caffeine was increased to 750 mgm. In this connection Mendel and Wardel (6) state: "The increase in the excretion of uric acid after adding coffee, tea or caffeine to a purine free diet seems to be proportional to the quantity of caffeine ingested." Data presented in charts 1 to 4 do not indicate a definite proportionality. Regardless of this increased urinary excretion there was also an increase in the uric acid content of the blood. This total increase in uric acid formation might have been brought about by:

1. Increased formation by oxidation of the caffeine (or its partially demethylated derivatives).

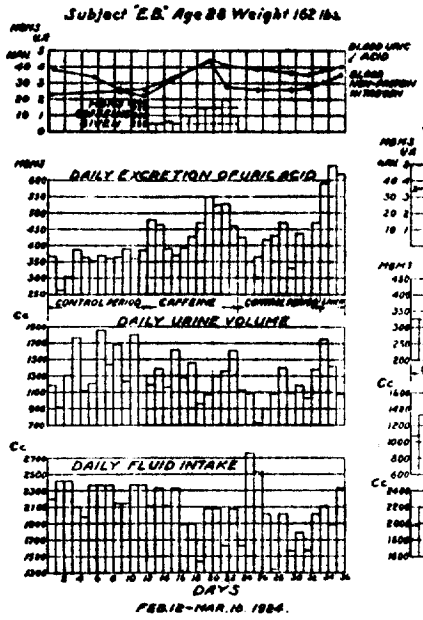
2. Increased nuclear activity, associated with a general stimulation of metabolism.

3. Either or both of the above, together with a non-compensating elimination.

[7] This question of the enzymic destruction of uric acid is much disputed. Folin, Berglund and Derick (4) state that: "The unique and characteristic high levels of uric acid in normal human blood are due to a lack of responsiveness on the part of the human kidney," and—"the uric acid destruction stops the instant the blood is removed from the living animal" (dog). Recent experiments lead Bollman, Mann and Magath (5) to think that: "The destruction of uric acid in the normal dog depends on the presence of the liver, as this process ceases as soon as the liver is removed." Some unpublished work by ourselves indicates that there is a 19 to 33 per cent destruction of uric acid, as determined by the direct colorimetric method, when sterile blood is kept at body temperature for a period of 2 to 8 days.

CHART 1.

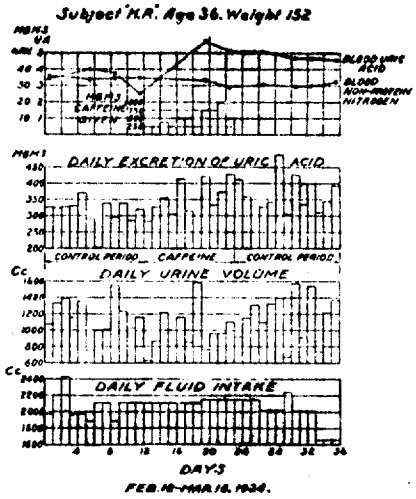
Subject "E.B." Age 28 Weight 162 lbs.



FEB.12–MAR.10. 1934.

CHART 2.

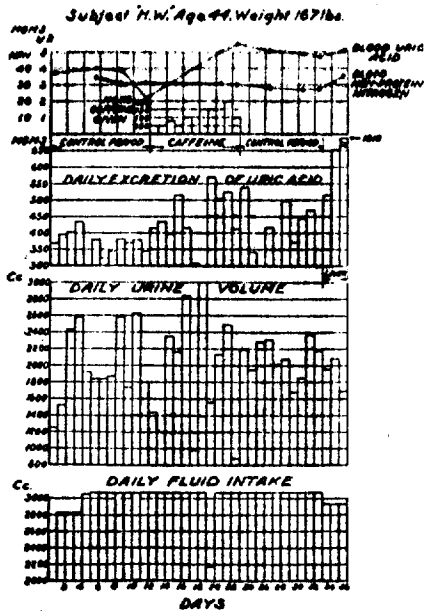
Subject "K.R." Age 36. Weight 152



FEB.16–MAR.16. 1934.

CHART 3.

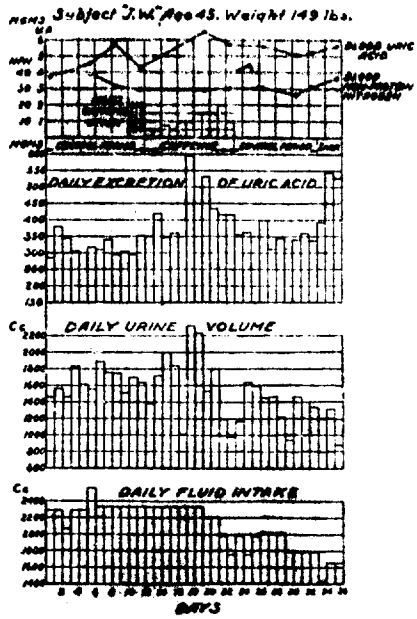
Subject "H.W." Age 44. Weight 167 lbs.



FEB.12–MAR.10. 1934.

CHART 4.

Subject "J.W." Age 45. Weight 149 lbs.



FEB.16–MAR.16. 1934.

First let us consider the possibility of the increased formation of uric acid from caffeine and theobromine. Mendel and Wardell (6) state that: "The increase in the amount of uric acid excreted under these conditions (using "Kaffee Hag," caffeine and tea) is equal to the quantity of uric acid which would be obtained by the demethylation and subsequent oxidation of 10 to 15 per cent of the ingested caffeine."

It will be recalled that the structure of the caffeine molecule differs from that of theobromine only by having the additional methyl group in position one. From the work of Krüger and his associates (7) it is probable that the methyl groups in the xanthine ring are decreasingly stable in the order 7, 1, 3. The methyl group in position 7 is common to both and if the work of Krüger and his associates be correct, there would be the possibility of a common decomposition product, 7 mono-methyl xanthine. If the stages in the oxidation of these substances are similar we should expect at least an equal amount of uric acid to be formed from molecular equivalents. With an additional methyl group in caffeine one should expect an extra stage of demethylation and we might therefore anticipate even less uric acid from oxidation of the caffeine molecule. In our experiments the doses of theobromine were almost double those of caffeine and if uric acid were being formed from direct oxidation of the molecule we should have further reason to expect it to be produced in larger amounts during the period of theobromine ingestion. The data presented in charts 5, 6, 7 and 8 indicate that such was not the case and that there was actually a decrease in the urinary excretion. The slight increase which was observed in the uric acid content of the blood can be accounted for by the diminished excretion. This diminished excretion and the accumulation of uric acid in the body tissues would suggest that theobromine had produced a "depressant effect" upon renal activity but that no increased formation of uric acid had been brought about by oxidation of the theobromine molecule. With the above mentioned structural similarities it is hardly reasonable to assume that the increase in the formation of uric acid in case of caffeine could be due to direct oxidation of its molecule.

As a second possible source of the increased formation of uric acid, at least following caffeine ingestion, we must consider the stimulating effects of this drug upon cellular metabolism. It has been found by Edsall and Means (8), Means, Aub and Du Bois (9) and others, that caffeine increases the basal metabolic rate from 5 to 30 per cent. With such a marked increase in cellular activity there should be a measurable increase in uric acid production.

The results of the part B experiments in which the uric acid was determined at intervals during a 24-hour period before and after caffeine ingestion, furnish additional evidence of this stimulation of nuclear metabolism, see charts 9, 10, 11. Although the blood uric acid remained constant it is

CHART 5.
Subject "E.B."

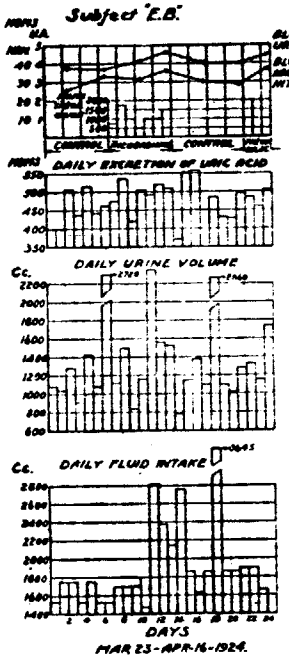


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CHART 7.
Subject "H.W."

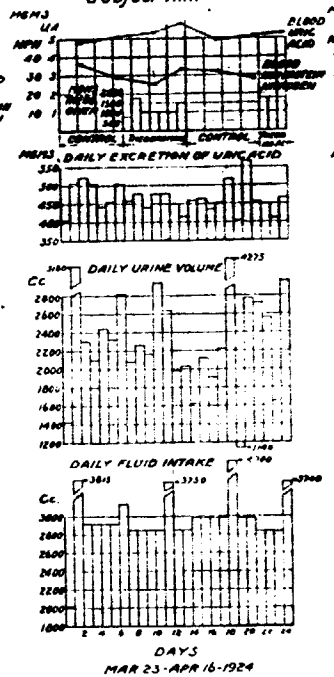

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CHART 6.
Subject "H.R."

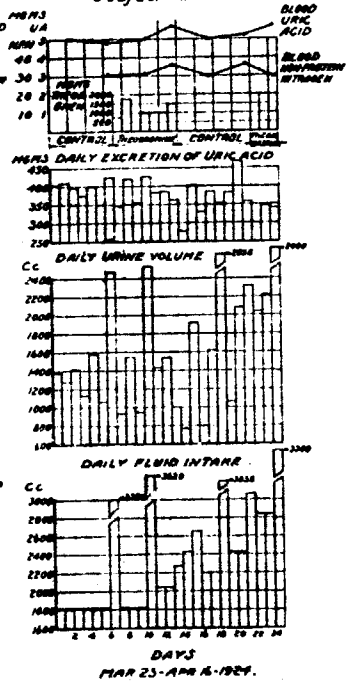

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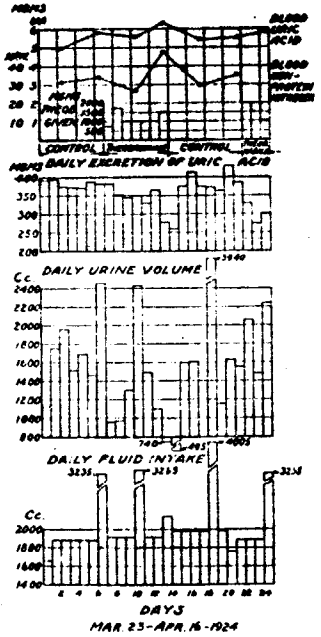
CHART 8.
Subject "J.W."



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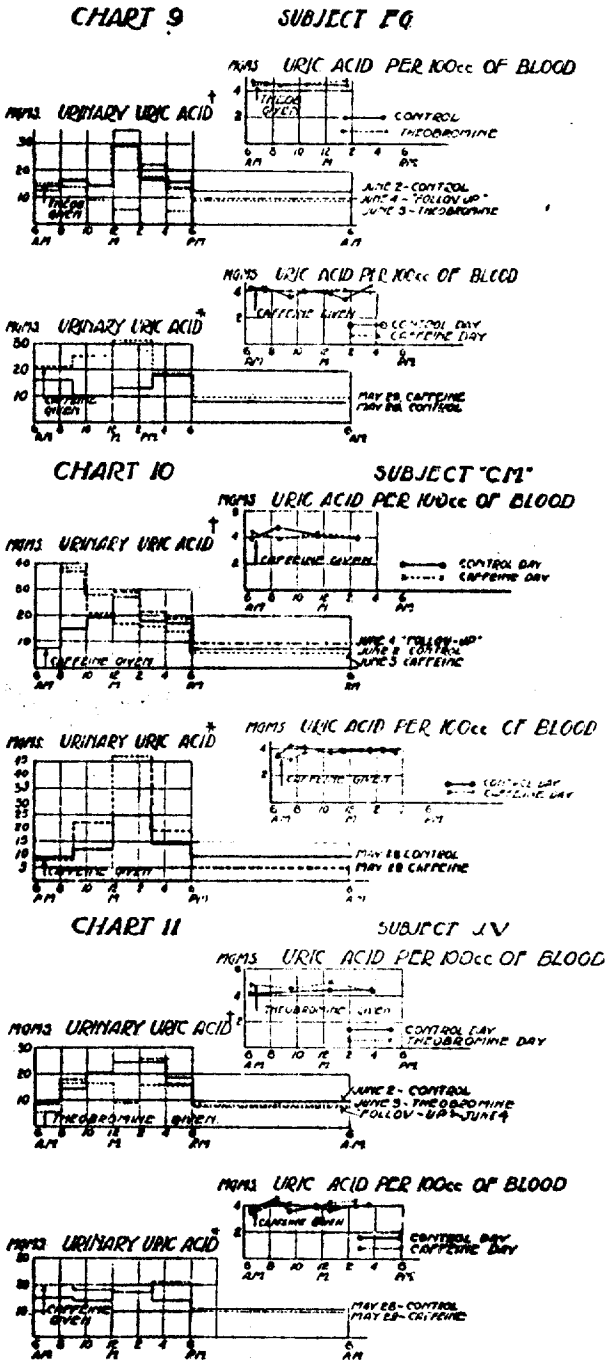

* In plotting the uric acid values, the total milligrams excreted were divided by 3, the number of hours in an experimental period (by 12 in the 6 p.m. to 6 a.m. period).

† In this set of observations the experimental period was reduced to 2 hours. In both instances the curves are drawn to represent the excretion in milligrams per hour.

evident that there was an increased excretion following the ingestion of caffeine. Let us consider subject F. G., chart 9. The uric acid excretion on the control day was 265 mgm., on the caffeine day it was 400 mgm. In case of subject C.M., chart 10, the respective values were 293 mgm. and 347 mgm.[8] In case of subject J.W., chart 11, values of 320 mgm. and 318 mgm. were obtained on the control and caffeine day respectively. As can be seen, the increased production and excretion of uric acid occurred within 8 to 12 hours, the maximum excretion being in all cases between the 5th and 7th hours. In every instance, except subject F. G. (on May 29) this initial increase in uric acid excretion was followed (during the night hours) by a decreased excretion. The theobromine results consistently showed diminished excretion.

In contrast to the apparent depressant effects[9] of the drugs very definite stimulation of uric acid excretion was obtained when the subjects were fed a high protein, high purine diet. On each of three days (34th, 35th and 36th, see charts 1, 3 and 4) three of the four subjects (E.B., H. W., J.W.) received from 200 to 225 grams of liver in addition to the regular diet. The fourth subject, H. R., did not care for liver and served as a control. In this period there was an immediate and very marked increase in the excretion of uric acid (see charts 1, 2, 3 and 4), with no significant change in the uric acid content of the blood.[10] However, the prompt increase in the excretions are indicative of there having been definite stimulation, i.e., in contrast to the effects of either caffeine or theobromine. This interpretation is in accord with the findings of Lewis, Dunn and Doisy (10) and Folin, Berglund and Derick (4).

There is the question as to whether this increased uric acid excretion has been produced by a parallel increase in the volume of urine. Morris and Rees (11) produced salt diuresis in rats and rabbits and state that: "As with urea, the output of uric acid is affected by the volume of urine excreted more than any other factor" and "There is no evidence of uric acid arising from increased kidney activity. The increased output is proportional to volume of urine excreted." On the other hand Mareš (12) reports that: "The supposed parallelism between the amount of

[8] Repeating the experiment three days later with this subject we obtained values of 299 and 382 mgm. for the control and "follow-up" days respectively and only 284 mgm. were excreted during the caffeine day, constituting the only exception. Edsall and Means (8) found a somewhat similar depression with a second dose of caffeine.

[9] Although charts 1, 5, 6, 7 and 8 indicate that there was a slight increased retention in the non-protein nitrogen of the blood, we do not feel that these results alone definitely indicate a depression but that when considered together with the excretion of uric acid, afford rather strong evidence of a depression in the excretion of organic substances.

[10] We do not mean to infer that prolonged ingestion of this high purine diet might not also have caused a greater damming back of the uric acid in the blood.

urine and the amount of uric acid excreted was not observed." Mendel and Brown (13) state that: "The incidence of large volume and increased uric acid output is accidental and by no means constant."

A study of the data obtained during the 12-day control period, prior to the administration of caffeine (see charts 1 to 4), indicates that under normal conditions[11] there is a relationship between the volume of urine and the quantity of uric acid excreted. On the other hand when the water intake, and subsequently the volume of urine excreted, was greatly increased there is absolutely no evidence of this relationship. This latter point was proven in these experiments by resorting to extreme water diuresis: subject E. B. on the 11th and 19th days, H. R. and J. W. on the 7th, 11th, 19th and 25th days, H. W. on 2nd, 12th, 19th and 25th days; see charts 5, 6, 7 and 8. As can be seen, although the fluid intake and the urine volume were greatly increased on each of these days, not one of the four subjects showed a parallel increase in the excretion of uric acid. These results suggest that uric acid is not passively excreted but that it is eliminated through specific activity of the renal cells.

Let us consider the correlation between this cellular activity, the acquisition of tolerance and the accumulation of uric acid in the blood. As mentioned, the results with caffeine (see charts 1, 2, 3 and 4) show an irregular but immediate increase in uric acid excretion following either an initial dose or a marked increase in dosage. Under the same conditions the initial dose of theobromine failed to give this immediate increase and on continuation even caused decrease in the uric acid excreted. We might reason that the observed increases in uric acid excretion, after caffeine, were caused indirectly, i.e., by the general metabolic stimulation previously mentioned, and that primarily the renal cells were reacting to the increased endogenous uric acid. Subsequently the excreting cells reacted to the foreign and more toxic substances (caffeine or various demethylated derivatives) resulting in a specific elimination of these substances to the partial exclusion of uric acid. The fact that the presence of large amounts of uric acid in the blood may stimulate the kidney to specific elimination has very recently been shown by Bollman, Mann and Magath (5). These workers found that when as much as 864 mgm. of uric acid were injected intravenously into a hepatectomized dog, 85 per cent could be recovered in the urine within 13 hours—a striking compensatory elimination, when the blood content exceeded its normal amount. The results obtained with theobromine are in accord with the above theory as to the acquisition of tolerance.

[11] Although the fluid intake was fairly constant there are many variations in the daily urine volumes. We have no measure of the water excreted through the skin, the amount of which would vary directly with external temperature, exercise, etc. The possible excretion of uric acid in the sweat is likewise an unknown factor. The relationship suggested is relative rather than absolute.

We have been unable to procure evidence to prove definitely that theobromine is devoid of any power of metabolic stimulation but our results indicate that there was at least no increased formation following its ingestion. The initial dosage seems simply to have allowed a normal excretion of uric acid. Subsequent doses apparently led to the same selective elimination of theobromine (or its methyl derivatives) and uric acid retention, just as was observed in case of caffeine.

CONCLUSIONS

1. The ingestion of either caffeine or theobromine tends to increase the concentration of uric acid in the blood.[12]

2. After caffeine ingestion there is evidence of an increase in uric acid production. After theobromine ingestion its formation seems to be unchanged, but its rate of excretion diminished. It seems probable that the increases in uric acid are not due to direct oxidation of these methylated xanthines.

3. Prolonged administration of either caffeine or theobromine seems to depress active excretion on the part of the kidney.

4. There exists no proportional relationship between the amount of drug given and the quantity of uric acid excreted.

5. Under normal conditions there exists a relationship between the volume of urine and the quantity of uric acid excreted, but under diuretic conditions (other than when produced by uric acid itself) no such relationship is evident. Apparently uric acid is actively excreted.

This work could hardly have been accomplished had it not been for the coöperation of ex-warden J. A. Johnston and the special efforts of Dr. L. L. Stanley, resident physician at the California State Prison, San Quentin.

[12] In the experimental work reported in this paper, part A was carried out February to April, 1924, and part B during May, 1925. Subsequent to the completion of the part A experiments there appeared articles by Bullmer, Eagles and Hunter (14) and by Hunter and Eagles (15) in which it was shown that whole blood contained material other than uric acid ("Substance X") which reacted with the Benedict (2) uric acid reagent to produce color. Very recently Benedict (16) has reported the presence of a second substance ("thiasine") in whole blood which also reacts with the uric acid reagent to produce color. The blood uric acid values (total color) reported in this paper are no doubt high and we are not in a position to state whether the observed changes in the uric acid of the blood were due to changes in "substance X," "thiasine" or uric acid per se. We hope to repeat a portion of this work, using an indirect method (precipitation with a silver salt) for the determination of uric acid.

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RELATION OF CAFFEINE AND COFFEE TO HUMAN EFFICIENCY.*

BY RALPH HOLT CHENEY.

Efficiency and diet occupy a more prominent position in the public mind to-day than in all previous history. At the moment, caffeine beverages, especially coffee, are lauded or berated vigorously regarding their value in the diet in relation to human efficiency.

Based upon "whims and guesses," coffee has been praised or condemned for centuries. What is the scientific evidence "pro" and "con?" Historically, we find a prohibition edict by Khaine Beg, Governor of Mecca in 1511, who started a campaign which condemned coffee as an inebriating beverage forbidden by the Koran because the people frequented coffee houses rather than the mosques. Coffee and coffee houses were taxed by European governments as a source of revenue. Later, coffee houses were prohibited by Charles II of England as "Seminaries of Sedition." Coffee houses have served as gathering places for public discussions of governmental affairs. They were the predecessors of the boulevard cafés of Paris and the coffee-house taverns of England. This English prototype appeared in the United States where the coffee houses of Boston and New York harbored the instigators of the Boston Tea Party and revolutionary propaganda.

In spite of six hundred years of agitation associated with coffee, its consumption has increased constantly from an insignificant amount to three billion pounds annually, and its use has spread from the Abyssinian hillsides to world-wide consumption. Mocha coffee from the original home of the plant now supplies less than 3% of the seeds (coffee beans) of this sweet-pulped, cherry-like fruit which was introduced in America in 1717. Its cultivation has changed the economic map of several countries. Brazil has two billion coffee trees, Colombia a quarter billion, and the success or failure of the crop is the chief factor in the economic situation in these countries. Eighty per cent of the total gold value of Salvadorean exports is coffee. Although the plant is foreign to American soil, no better coffee is marketed to-day than the Colombian or Brazilian Santos from the Uplands where it is prepared by the most modern methods.

The United States consumes over one-half of all the coffee exported from the cultivated areas. New York City requirements annually total seventy million pounds, for which we pay thirty million dollars. Space limitation causes me to refer you to a book, "Coffee" (1), published by the New York University Press, for botanical characteristics; and also, for further details of the interesting, romantic history and economics of the plant and its beverage.

Undoubtedly, more scientific experimentation on the subject of caffeine and coffee effects has been conducted during the last twenty-five years than in all previous time. The results of such investigations are the only justifiable bases from which we may make our deductions regarding the physiological effects of the coffee as such, and of its individual ingredients.

Many investigators have reported that caffeine and caffeine beverages are undesirable for young children, neurotics, sufferers from severe arteriosclerosis, gastric hyperacidity, and other abnormal conditions. Likewise, the administration of caffeine for therapeutic purposes, such as stimulation of the coronary circulation in cardiac deficiency, are well known. May I make it perfectly clear, however, that the data presented in this paper is not concerned with the allergic effects of caffeine nor of the coffee beverage upon pathological conditions. This paper does not deal with its therapeutic value. All statements and figures refer entirely to *normal* physiology. The experimental animals and human subjects employed in the investigations were selected purposely from individuals of as nearly average good health as could be determined readily. In the caffeine work, the Merck product was used, and in the coffee work, the beverage was prepared by a rapid method (drip or vacuum) from recently torrified Colombian and Brazilian, high-grade beans.

Isotonic and isometric records of striated muscle fatigue curves of homologous muscles were made under non-treated and caffeine-treated conditions. The method and period of treatment were maintained uniformly throughout the series. Detailed discussions may be found in the writer's earlier papers, especially on the animal gastrocnemius behavior (2, 3).

Although Herxheimer (4) found that caffeine was of no benefit to the athelete, and P. T. Osborne states that caffeine actually interferes with the best muscular effects, the experimental data in this paper are more in agreement with H. C. Wood, Jr., (5) and others whose results offer definite evidence of improved muscular function.

* New York Branch, A. PH. A.—See page 70, January JOURNAL.

An extended series indicated that better physiological coördination of the motor mechanism was obtained in the striated muscle action of the frog under the influence of low concentration of caffeine. According to the data presented in the August 1932, issue of the *Journal of Pharmacology and Experimental Therapeutics*, the optimum dosage (3) for these muscles for delaying fatigue was 0.1 mg. per Gm. body weight. Similar studies using the coffee beverage as such, in lieu of the purified caffeine, showed a parallel response for the gastrocnemius muscle.

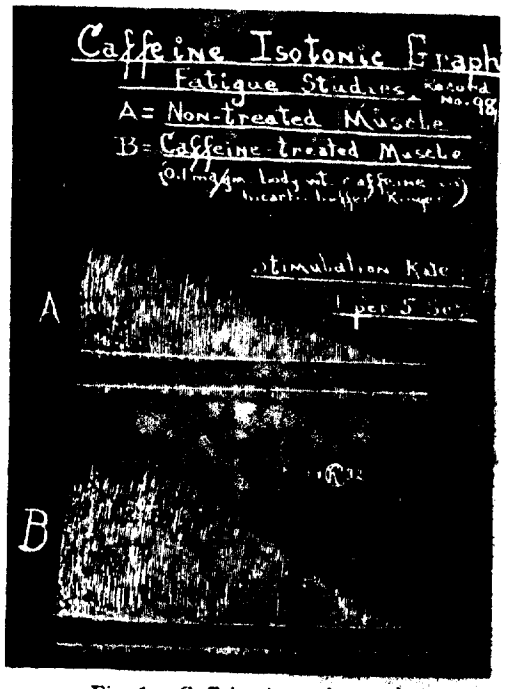

Fig. 1.—Caffeine isotonic graph.

A mathematical analysis of these fatigue curves in computing the XY values (Time *to* one-half fatigue × Height *at* one-half fatigue) demonstrated a greater XY value in both the caffeine and coffee curves as compared with the normal, non-treated result.

Under uniformly controlled circumstances, a study of the physical fatigue in human male subjects was attempted. The variable factor was the treatment; namely, the consumption of equal volumes of hot water, black coffee, or hot water plus caffeine (equivalent to the amount in the coffee) prior to the fatigue test involving a weight-lifting exercise every ten seconds to complete fatigue. Additional non-treated, starch capsule plus hot water, or plain hot water days were completed in order to correct for any practice, accommodation or experience factor which may have developed during the experimental tests. The Work Done was computed by the simple formula: WD = Weight (Gm.) × Height lifted (cm.) × Number of lifts. The result in gram-centimeters showed a sequence of an increasing amount of Work Done in the order of the non-treated or hot water day (least), coffee day, caffeine day (most). Attention was given primarily to the following physiological factors:

1. Blood-pressure variations: Systolic and diastolic.
2. Respiration: Rate and depth by pneumographic record.
3. Time required for a complete recovery of all factors after fatigue.

As compared with the non-treated fatigue values, the blood pressure and pulse variations were negligible; that is, their variation under the treated conditions was similar to their variation under the non-treated conditions. The increased respiratory rate and depth in the coffee instance indicated an increased oxygen consumption.

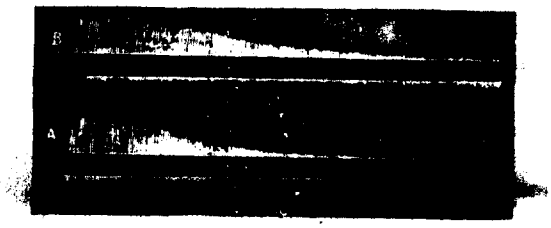

Fig. 2.—Caffeine isometric graph.

Fatigue Studies: Record No. 888.
A = *Non-treated* muscle; B = *Caffeine-treated* muscle. (0.1 mg./Gm. body weight caffeine in bicarb. buffer-Ringer) stimulation rate: 1 per 5 sec.

Several years ago (6), I conducted a series of tests to determine blood-pressure variations *without fatigue*, as influenced by caffeine and by coffee in comparison with normal behavior. This work has been repeated recently as an additional series. Similar and additional substantiating data were obtained. The accompanying graph separates the primary and secondary coffee effects.

and indicates the fact that other substances are involved besides caffeine in the total coffee effects. Note that the caffeine or coffee increased the blood pressure only a few millimeters in excess of the ordinary luncheon (food) effect.

Although caffeine has been reported as a vaso-dilator of cerebral vessels, investigations (7) by Dr. P. G. Denker of Bellevue Hospital, caused him to conclude that caffeine definitely lowers the cerebrospinal pressure for half an hour. This fact is suggested by some workers as a possible explanation of the recognized relief induced by coffee in instances of mild hypertensive or nervous headaches, cerebral concussion, fractured skull and brain tumor. Sollman and Pilcher (8) reported in 1911 that caffeine causes vaso-dilation of the cerebral vessels. Wiechowski (9) had reported similar results in 1902 and claimed the dilation was due to a direct decrease in the tonus of the intracranial vessels. Such action with a rigid cranium would force out the cerebrospinal fluid and increase its pressure. Denker obtained the reverse results. With reference to the decreased cerebrospinal pressure it is significant that caffeine diuresis through glomerular action, involves primarily only an increase in the water content of the urine. This water loss must be lost necessarily from the body tissues through the lymph and blood. It must accomplish

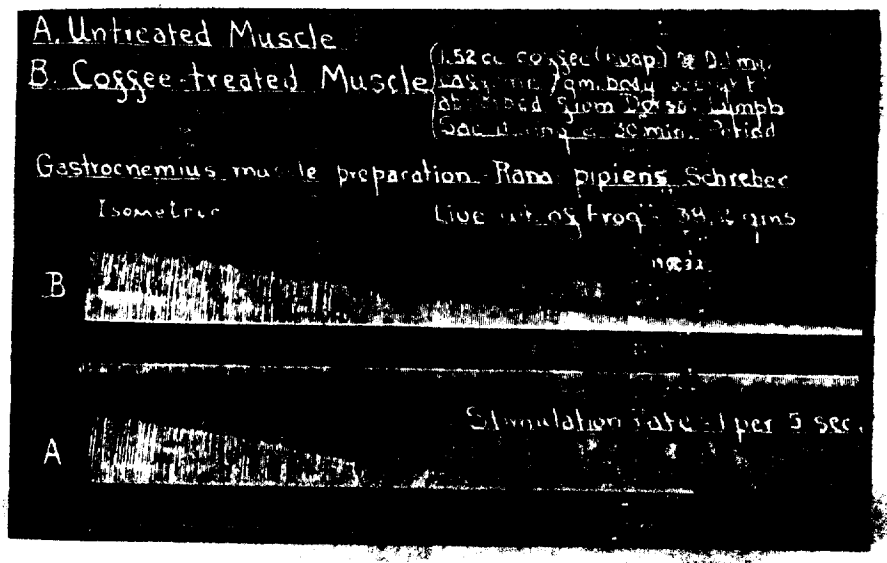

Fig. 3.—Coffee effect on muscular fatigue.

automatically, a reduction in brain volume with a consequent decrease in cerebrospinal fluid pressure.

The increased respiratory rate and depth, referred to earlier in connection with the fatigue as influenced by caffeine and coffee, is related also to an intercranial pressure. In 1913, Dixon and Halliburton (10) noted that reduction of carbon dioxide in the blood decreases the rate of cerebrospinal fluid secretion. Therefore, respiratory stimulation by caffeine or coffee, resulting in increased rate of carbon dioxide ventilation from the lungs, would decrease the rate of cerebrospinal fluid secretion. Apparently, caffeine lowers intracranial pressure by means of its combined pharmacologic actions rather than by any specific, single effect.

Dr. A. L. Winsor (11) of Cornell University, reported last October that coffee increases the salivary secretions in volume per unit of time. Such stimulation automatically improves digestion by making a greater enzymic mass available.

Professor Hollingworth's experimental series (12, 13) on the mental effects of caffeine and coffee are the best available studies on this phase of the subject. He reports a clearly distinguishable mental stimulation as evidenced by greater accuracy in the performance of work done.

Recently at Long Island University, a simplified psychodometer was devised to measure the reaction time in man with a visual-mental-physical coördination test. Quantitative data on

the relationship of caffeine and coffee separately to the normal reaction time were compiled. The subject was required to recognize a visual stimulus (light color), interpret it, and perform a muscular act in accordance with the color of the particular stimulus involved. Caffeine and coffee were each more effective in reducing the total reaction time in women than in men. The sequence in both sexes was as follows:

1. Non-treated tests required the longest reaction time.
2. Coffee—intermediate.
3. Caffeine-treated tests required the shortest reaction time.

In various physiological effects studied regarding muscular action, fatigue delay, respiration, pulse and mental effects, the sequence of minimum to maximum physiological stimulation is generally non-treated, coffee-treated, caffeine-treated. The absence of injurious effects in the majority of cases suggests the importance of optimum consumption. Excessive stimulation can be produced, but was not apparent in the amounts administered in the experiments upon normal animals and man, although the quantities given were in excess of average consumption. The absence of after-effects and accumulative effects is associated doubtlessly with the two primary facts:

1. Caffeine is combined with other chemical substances in coffee.
2. Caffeine itself loses CH_3 groups in the body and is excreted within a few hours in the urine as mono- and dimethyl xanthine.

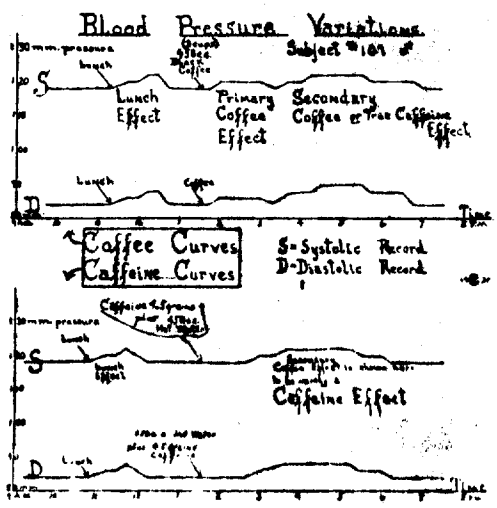

Fig. 4.—Blood-pressure variations: caffeine vs. coffee effects.

Likewise, Gutman (14), in agreement with this conception, observed that caffeine and coffee effects are not the same in abnormal persons (patients) showing allergic manifestations to coffee. Whereas caffeine may cause definite physiological disturbances, coffee may be well tolerated. Moreover, the action of coffee is not due solely to caffeine, but to the many compounds organized during the roasting process, and which are present in both decaffeinated and ordinary coffees.

Caffeine and coffee increase blood pressure very few millimeters, respiration and oxygen consumption slightly, and stimulates the brain and other parts of the central nervous system. These stimulants also delay fatigue and lower the cerebrospinal pressure. Caffeine is less effective in the coffee beverage than separately. Neither complex, caffeine nor the coffee beverage as such, can replace nutritive metabolism in the normal health of the tissues. It has been reported that both complexes increase basal metabolism; hence, food should always be taken regularly at the customary periods if the reserve forces of the body, which are reduced by increased metabolism, are to be replenished properly. Nevertheless, many people labor under the misconception that coffee has the ability to replace food merely because its stimulating action persists for several hours after consumption. It is an aid to functional efficiency, not a substitute for body fuel.

The variations from the normal due to coffee stimulation are slight and, in the reasonable amounts consumed ordinarily, are far below the maximum physiological disturbances, for the correction of which the body is equipped by nature to adjust itself without injurious effects.

Apparently, the delicate correlation between the increased blood flow and the increased respiratory rate accomplishes, in a fortunate sequence, both the more rapid removal of fatigue products from the body tissues to the blood, and, the greater purification (oxidation) of the blood

volume per unit time in the lungs. Fatigue, due to the accumulation of waste products in the tissues, is correspondingly delayed. Caffeine separately or in the coffee beverage, stimulates the circulation and respiration. The increased activity of one of these system without a parallel response by the other, would be injurious. The stimulation of both synchronously, produces or "sets" the body metabolism at a higher level of physiological efficiency. That the body readily adjusts itself to the level of performance without injury is evidenced by the fact that the recovery time of the fatigue is practically identical with conditions subsequent to normal fatigue.

The bulk of available experimental data seems to indicate that the majority of the population may take advantage of this beneficial aid to body metabolism. Every debatable question has its "pro" and "con." Each of us has a caffeine and coffee tolerance which must be determined individually, and our consumption governed accordingly.

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Preliminary Communication

COFFEE-DRINKING AND CANCER OF THE LOWER URINARY TRACT

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Summary Interviews were conducted with a population-based series of patients with cancer of the lower urinary tract and controls drawn from the same population. An unanticipated finding is association of the disease with coffee-drinking. The relative risk for coffee-drinkers, compared to non-drinkers, is 1·24 among men and 2·58 ($P<0·05$) among women. The association is strong in the sex-age groups in which the associations of the disease with cigarette-smoking and occupation are not strong.

INTRODUCTION

A CASE-CONTROL study was conducted to quantitate the roles of cigarette-smoking and occupational exposures in the ætiology of cancer of the lower urinary tract. An unanticipated finding, described here, is the association of the disease with coffee-drinking.

METHODS

A previous report [1] from this study describes the sources of data. During a recent 18-month period, in an area in eastern Massachusetts including Boston, an attempt was made to identify all residents aged 20–89 newly diagnosed with histologically confirmed transitional or squamous-cell malignancy of the renal pelvis, ureter, bladder, or urethra. 668 cases were ascertained. Since the bladder was involved in more than 90% of the cases, the term "bladder cancer" will be used for brevity. A random sample of 510 of the patients was selected and 468 (92%) were interviewed. For each case a person of the same sex and age was selected at random from an age and sex stratified sample of the population of the study area. A random sample of 552 of these persons was designated as controls and 498 (91%) were interviewed. Only two questions were asked about coffee-drinking: "Do you drink coffee?" and if the subject answered yes "About how many cups per day?"

Data from this study reveal a consistent association of bladder-cancer risk with cigarette-smoking and an association with occupational exposures which is essentially restricted to men aged less than 75. In view of these findings, all estimates of risk for coffee-drinkers in this paper are controlled for cigarette-smoking and among men, for occupation. Among the 468 interviewed cases, 445 (345 men, 100 women) provided information adequate for assessment of cigarette-smoking, occupational experience, and coffee-drinking. Of the 498 interviewed controls 451 (351 men, 100 women) provided adequate information on the three variables.

To control for cigarette-smoking, three categories of maximum daily cigarette consumption were used: non-smokers, smokers of up to one-half package per day, and smokers of more than one-half package per day. For occupational exposures, two categories were used: "high risk" (applied to men ever employed in an industry where increased risk was suspected a priori and supported in the present study) and "other" (applied to men never so employed). For descriptive purposes, observed and "expected" numbers of cases are presented in various categories. The expected numbers result when the cases are distributed with respect to coffee-drinking as are the controls. All expected numbers have been adjusted for the same variables as the risk estimates. The relative risks and their 95% confidence-limits were derived using Sheehe's modification [2] of Woolf's technique. When the confidence-limits of a relative risk do not include 1·00, the risk is "statistically significantly" elevated with a probability due to chance of less than 5%.

RESULTS

Table I shows the overall association of bladder-cancer risk with coffee-drinking. For both sexes, drinkers of less than one cup of coffee per day have lower bladder-cancer risk than any other "exposure" category. Although there is no consistent dose-response relationship for either sex, among women the risks are higher in the higher-exposure categories. Among men, overall, controlling for age as well as cigarette-smoking and occupational experience, the relative risk for "coffee-drinkers" (persons who drink one or more cups per day) is 1·24 (95% confidence-limits 0·80–1·93), compared to a risk of 1·00 for "non-drinkers" (persons who drink no coffee or less than one cup per day). Among women, overall, controlling for age as well as cigarette-smoking, the relative risk is 2·58 (1·80–5·10).

TABLE I—OBSERVED AND EXPECTED NUMBERS OF MALE AND FEMALE CASES AND RELATIVE RISK ACCORDING TO DAILY COFFEE CONSUMPTION

Cups per day	Men			Women		
	Obs.	Exp.*	R.R.‡	Obs.	Exp.*	R.R.‡
<1	29	41·2	1·00	9	22·9	1·00
1	86	78·8	1·34	19	29·4	1·60
2, 3	146	153·1	1·19	50	26·4	3·76
4+	84	71·9	1·31	22	19·4	2·19
Total	345	345·0	..	100	100·1	..

* Controlled for age, cigarette-smoking, and occupation
† Controlled for age and cigarette-smoking.
‡ Relative to a risk of 1·00 among non-drinkers and drinkers of less than one cup of coffee per day.

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TABLE II—OBSERVED AND EXPECTED NUMBERS OF CASES WHO NEVER SMOKED NOR EVER HAD A HIGH-RISK OCCUPATION AND RELATIVE RISK ACCORDING TO DAILY COFFEE CONSUMPTION, SEXES COMBINED

Cups per day	Cases		
	Obs.	Exp.*	R.R.†
<1	20	21·1	1·00
1	31	24·7	2·18
2, 3	37	36·9	1·84
4	12	7·4	2·60
Total	90	90·1	..

* Controlled for age and sex.
† Relative to a risk of 1·00 among non-drinkers and drinkers of less than one cup of coffee per day.

In addition to controlling for the effect of cigarette-smoking, as above, the effect can be eliminated from the association of coffee-drinking and bladder cancer by restricting the analysis to non-smokers (persons who stated they had not smoked 100 cigarettes during their life). Among 66 non-smoking male cases, there were 7 who did not drink coffee; the expected value is 12·8—a relative risk for coffee-drinkers of 1·41 (0·65–3·06). Of 49 non-smoking female cases, 5 did not drink coffee; the expected value is 13·9—a relative risk for coffee-drinkers of 3·47 (1·26–9·50). Table II shows the dose-response relationship for the sexes combined. In addition to all smokers, all persons ever employed in a high-risk occupation are excluded from table II. For persons who neither smoked nor worked in a high-risk occupation, the relative risks are higher than those shown in table I and highest risk is present in the highest exposure group.

The associations of bladder-cancer risk with cigarette-smoking and occupation were found to be stronger for men than for women and for younger than for older persons. It therefore seemed worth while to explore the association of bladder cancer with coffee-drinking according to sex and age (tables III and IV). The association is stronger for women than for men and, among men, increases with age. That is, the association with coffee-drinking is most distinct in those age and sex groups in which occupation and cigarette-smoking play the smallest role.

TABLE III—OBSERVED AND EXPECTED NUMBERS OF MALE CASES AND RELATIVE RISK AMONG COFFEE-DRINKERS, BY AGE

	Cups of coffee per day in following age-groups (years)					
	20–59		60–74		75–89	
	<1	1	<1	1	<1	1+
Cases observed	11	86	11	146	7	84
Cases expected*	6·0	90·5	20·3	137·7	15·1	75·9
Relative risk†	1·00	1·68	1·00	1·38	1·00	1·98

* Controlled for age, cigarette-smoking and occupational exposure.
† Relative to a risk of 1·00 among non-drinkers and drinkers of less than one cup of coffee per day.

TABLE IV—OBSERVED AND EXPECTED NUMBERS OF FEMALE CASES AND RELATIVE RISK AMONG COFFEE-DRINKERS, BY AGE

	Cups of coffee per day in following age-groups (years)					
	20–59		60–74		75–89	
	<1	1	<1	1	<1	1+
Cases observed	2	35	2	34	5	24
Cases expected*	6·0	27·0	9·3	26·7	5·7	23·3
Relative risk†	1·00	3·68	1·00	4·49	1·00	1·11

* Controlled for cigarette-smoking.
† Relative to a risk of 1·00 among non-drinkers and drinkers of less than one cup of coffee per day.

Since the study involved a series of cases from a defined population, and since the controls reflect the coffee-drinking habits of that population, the population attributable risk per cent[4] of bladder cancer associated with coffee-drinking can be estimated. This measure reflects both the proportion of the population exposed to a risk factor and the increased risk among them. For men, the population attributable risk per cent for coffee-drinking is 24%, suggesting that about a fourth of bladder cancer among men could be due to coffee-drinking. For women, the estimate is 49%, suggesting that about half of their disease experience might be so caused.

DISCUSSION

Takahashi[5] and Stocks[6] correlated national mortality-rates for several cancers with estimates of concurrent per caput coffee consumption. No correlation was found for bladder cancer. However, this approach ignores the 20-year induction period likely for bladder cancer. Furthermore, the present data suggest that in a country such as the United States, with typical per caput coffee consumption, not more than a third of the disease is likely to be attributable to this cause. National differences in the frequency of factors responsible for the other 70% of the disease are likely to make correlations based on total rates unrewarding. Another approach is to relate secular changes in bladder-cancer incidence and coffee consumption within a single country. From about 1945 to about 1960 there may have been a genuine slight rise in the incidence-rate of bladder cancer in the United States.[7] During this period there was about a 20% decline in per caput coffee consumption.[8] However, the data on coffee consumption are neither sex nor age specific, and the overall reduction may have resulted from a shift in the age structure of the population.

Previous observations are consistent with an association between bladder-cancer risk and coffee-drinking. Caffeine, a major constituent of coffee, is mutagenic in lower organisms, *Drosophila*, and human cells, in vitro.[9] A known carcinogen, 3,4-benzpyrene, has been found in soot from coffee-roasting plants.[10] Of 25 guineapigs subjected to an aerosol of this soot, 2 developed carcinomas and 5 developed "pre-malignant" lesions of the bladder.[11] In a case-control study[12] carried out in New Orleans in 1958–64 an association between bladder cancer and coffee-drinking was found which was not statistically significant overall. Nevertheless, there was a higher proportion of coffee-drinkers among cases than among controls in each sex-race group other than White women. It can be estimated from the New Orleans data that the crude relative risk (unadjusted for age or cigarette-smoking) of bladder cancer among coffee-drinkers is 1·81, compared to 1·00 among non-drinkers.

Two observations on the present data detract from the association between bladder cancer and coffee-drinking. These are the inconsistent dose-response relationship and the low strength of the association among men. However, the study was not designed to evaluate the role of coffee-drinking and the relevant information is neither as precise nor as detailed as it might be. It may be expected that there has been some random misclassification of cases and controls with respect to coffee-drinking habits. The effect of this would be to obscure any dose-response relationship and to weaken the overall association.

The conclusion reached is only that the relationship between coffee-drinking and bladder cancer warrants investigation. Detailed information on amounts and types of coffee consumed should be useful since decaffeinated and instant coffees contain less caffeine than regular coffee. Information on substances ingested with coffee, especially artificial sweeteners, should also be obtained, although cyclamates, at least, are unlikely to be responsible for any association which may have existed during the 1960s.[13]

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A STUDY OF THE METABOLISM OF THEOBROMINE, THEOPHYLLINE, AND CAFFEINE IN MAN*

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Previous studies (1, 2) have shown that after the ingestion of caffeine or theophylline by man there was an increased urinary excretion of material which gave a blue color with the alkaline arsenophosphotungstate reagents used in the determination of uric acid. Myers and Wardell (3) and Buchanan, Christman, and Block (1) presented evidence that this extra color was caused by methyluric acids rather than by an increase in true uric acid excretion. When similar amounts of theobromine were taken, there was no increase in the excretion of chromogenic material.

Subsequent work (2) demonstrated the presence of 1-methyl and 1,3-dimethyluric acids in urine after the administration of caffeine and theophylline. The excretion of 7-methylxanthine, 1-methylxanthine, and 1,7-dimethylxanthine after the ingestion of caffeine has recently been reported by Weissmann *et al.* (4).

The present investigation concerns the identification and quantitative determination of the methyluric acids and methylxanthines excreted in the urine of man after the ingestion of theobromine, theophylline, and caffeine. 65 to 75 per cent of 1 gm. oral doses of these compounds were accounted for in the urine as methyluric acids or methylxanthines.

EXPERIMENTAL

Two individuals were placed on a diet free from coffee, tea, and cocoa. 24 hour control urine samples were collected, weakly acidified, and preserved by freezing. A 1 gm. sample of theobromine, theophylline, or caffeine was taken over a 4 hour period as two 500 mg. doses. Subsequent 24 hour urine samples were collected over a 3 day period and kept frozen until analyzed.

Chromatography—5 ml. aliquots of the urine samples were passed through a Dowex 2 anion exchange resin column which had been equilibrated with 0.1 N HCl. Washing the column with water removed the methylxanthines which are not strongly absorbed under these conditions. Uric acid and

* This material was taken in part from a thesis submitted to the Horace H. Rackham School of Graduate Studies, University of Michigan, by Herbert H. Cornish in partial fulfilment of the requirements for the degree of Doctor of Philosophy (1956).

methyluric acids were retained on this column and subsequently eluted with 0.01 N HCl. This fraction will be referred to subsequently as the uric acid fraction.

Both the water washings (methylxanthine fraction) and the 0.01 N HCl eluate (uric acid fraction) were evaporated to dryness, dissolved in a minimal volume of weak alkali, and each was chromatographed on Whatman No. 1 filter paper by the ascending technique (solvent: *n*-butanol, water, acetic acid in a 4:1:1 ratio by volume). Known samples of the various methylxanthines and methyluric acids were chromatographed simultaneously to determine the R_F values for these compounds. After chromatography it was possible to render the methylxanthines and methyluric acids visible under ultraviolet light (5).

The strips containing these compounds were eluted with 10 ml. of 0.1 N HCl in the manner described by Dent (6). The ultraviolet absorption spectrum of each eluate was determined in acid (0.1 N HCl) and alkaline (0.05 N NaOH) solution by means of a Beckman model DU spectrophotometer. Light absorption measurements were made against a blank eluate from paper chromatographed in the same solvent.

Since all the methyluric acids which can be formed from theophylline are chromogenic, 0.2 ml. portions of the urine samples were chromatographed directly on paper with a solvent composed of methanol, benzene, *n*-butanol, and water (2:1:1:1 by volume). After detection of the absorbing areas under ultraviolet light, the chromatograms were cut into appropriate strips, the methyluric acids eluted with urea-cyanide solution (7), and the colorimetric determination made in the usual manner.

Silver Salt Precipitation—Another method for the identification of the methylxanthines was based on the solubility of their silver salts at various pH values. 5 ml. of an aqueous solution containing from 50 to 100 γ of the methylxanthine were adjusted to the desired pH and 5 ml. of 5 per cent silver nitrate were added. The tube was stoppered and allowed to stand for 10 to 15 minutes with occasional shaking. After centrifugation at moderate speed for 5 minutes, the supernatant fluid was decanted and the tube allowed to drain. The methylxanthine was reconstituted by shaking the precipitate vigorously for 5 minutes with 5 or 10 ml. of 10 per cent NaCl in 0.1 N HCl. After centrifugation, its concentration was determined in the supernatant solution by measurement of the optical density at 270 mμ. All ultraviolet absorption measurements were made against a reagent blank carried through the entire silver precipitation procedure.

Table I records the percentages of the methylxanthines which were precipitated by silver nitrate at various pH values and then reconstituted into the acid-NaCl supernatant fluid. It can be seen that mixtures of

some methylxanthines can be separated by this procedure. For example, from a mixture containing theobromine (3,7-dimethylxanthine), 3-methylxanthine, and 7-methylxanthine, only the latter compound is precipitated by silver nitrate at a pH of 1 to 2. Adjustment of the pH of the supernatant fluid with alkali and acetate buffer (8) to a pH of 5.5 and addition of more silver nitrate will precipitate 95 per cent of the 3-methylxanthine, leaving the theobromine in solution. This type of analysis has been applied to the xanthine fractions from resin columns and to eluates from paper chromatograms. When known amounts of 3-methylxanthine, 7-methylxanthine, and 3,7-dimethylxanthine (theobromine) were added to control urine samples, each could be determined by this procedure with an accuracy of 90 to 95 per cent.

Results

Methylxanthine and Methyluric Acid Excretion in Urine of Man after Ingestion of Theobromine—After the ingestion of 1 gm. of theobromine by each of two individuals, there was no increase in the excretion of either true uric acid or material giving residual color as determined by the uricase procedure (7). Paper chromatography of the uric acid and methylxanthine fractions obtained from the anion exchange column indicated the presence of a number of theobromine metabolites in the fractions from both the 0 to 24 hour and 24 to 48 hour samples.

In the chromatogram of the uric acid fraction, ultraviolet light-absorbing areas corresponding to uric acid (R_F 0.18) and 7-methyluric acid (R_F 0.35) were found. 3-Methyluric acid, another possible metabolite of theobromine, was not detected on this chromatogram. After elution from the paper, further identification of 7-methyluric acid was made by its characteristic absorption spectra in acid and alkaline solution and its failure to give a color reaction with alkaline phosphotungstate solutions. As shown in Table II, relatively small amounts of 7-methyluric acid are excreted during the 48 hours following theobromine ingestion.

The paper chromatograms of the methylxanthine fraction had ultraviolet-absorbing areas at R_F values of 0.41, 0.48, and 0.60 corresponding to those of 7-methylxanthine (0.41), 3-methylxanthine (0.48), and theobromine (0.60), respectively. The eluted material with an R_F of 0.60 had absorption maxima at 271 mμ in acid (0.1 N HCl) and 273 mμ in alkaline solution (0.05 N NaOH). These values agree well with those for theobromine in acid (272 mμ) and in alkali (274 mμ). The theobromine excreted per 48 hours (Table II) was calculated from the optical density measurements of the acidified eluates.

Since the areas containing 7-methylxanthine and 3-methylxanthine overlapped to some extent, they were eluted from the paper as a single fraction

and separated by fractional precipitation as silver salts at pH 1.0 and 6.0 (Table I). Further identification was made by the characteristic shift in absorption maxima when the medium was changed from acid (0.1 N HCl to alkali (0.05 N NaOH), 3-methylxanthine shifting from a peak of 270 in acid to 275 mμ in alkaline solution, 7-methylxanthine from 267 to 290 mμ. The values reported in Table II for the 48 hour excretion of 3- and 7-methylxanthine have been calculated from the optical density values in acid solution.

Methylxanthine and Methyluric Acid Excretion in Urine of Man after Ingestion of Theophylline—The procedure for the determination of true uric acid (7) indicated that, after the ingestion of theophylline, there was a sharp increase in the excretion of chromogenic substances that were not

TABLE I

Silver Salt Precipitation of Methylxanthines from Water Solutions at Various pH Values

Concentration range, 1.0 to 2.0 mg. per 100 ml.

Compound	Per cent recovery		
	pH 1.0 to 2.0	pH 5.0 to 6.0	pH 10.0 to 12.0
1-Methylxanthine	30	91	94
3-Methylxanthine	0	95	95
7-Methylxanthine	92	93	97
1,3-Dimethylxanthine	0	93	84
1,7-Dimethylxanthine	74	92	97
3,7-Dimethylxanthine	0	5	50–80
1,3,7-Trimethylxanthine	0	0	0

destroyed by uricase. This increase could be attributed to the presence of one or all of the chromogenic methyluric acids (1-methyl, 3-methyl, and 1,3-dimethyl). After separation by direct paper chromatography, as previously described, the colorimetric analyses indicated that 1-methyluric acid and 1,3-dimethyluric acid were present in considerable quantity (Table II).

A preliminary separation of the methylxanthines from the uric acids was made on Dowex 2 anion exchange resin, as previously described. 3-Methylxanthine (R_F 0.48) has absorption maxima in acid and alkaline solutions at 270 and 275 mμ, respectively. The fraction separated from urine at an R_F value of 0.45 to 0.59 had absorption maxima at 267 and 275 mμ in acid and alkaline solutions. The presence of a small amount of 1-methylxanthine (R_F 0.55) in this fraction cannot be ruled out, although the two maxima at 242 and 277 mμ, characteristic of 1-methylxanthine in

alkaline solution, were not observed. The fraction eluted from the paper with an R_F of 0.70 (0.65 to 0.75) had absorption maxima in acid and alkaline solutions at 267 and 273 mμ, respectively, as compared to 271 and 275 mμ for a known sample of theophylline (R_F 0.70). The values for the 3-methylxanthine and theophylline (Table II) were calculated from the optical density measurements of the corresponding acid eluates.

Methylxanthine and Methyluric Acid Excretion in Urine of Man after Ingestion of Caffeine—After the ingestion of 1 gm. of caffeine, there was a

TABLE II

Methylxanthine and Methyluric Acid Excretion during 48 Hour Period after Ingestion of 1 Gm. of Designated Methylxanthine

All the values are expressed as mg. of the administered compound required to give the excretory product.

Compound excreted	Compound ingested					
	Theobromine		Theophylline		Caffeine	
	Subject H. C.	Subject F. M.	Subject H. C.	Subject F. M.	Subject H. C.	Subject F. M.
Theobromine (3,7-dimethylxanthine)	124	117				
3-Methylxanthine	182	215	155	111		
7-Methylxanthine	280	281			90	40
7-Methyluric acid	42	36				
Theophylline (1,3-dimethylxanthine)			87	111		
1-Methyluric acid			186	186	254	304
1,3-Dimethyluric acid			382	318	142	46
Caffeine (1,3,7-trimethylxanthine)					11	12
1-Methylxanthine					182	199
1,7-Dimethylxanthine					74	19
Total	628	649	810	726	753	620

marked increase in the excretion of non-uric acid chromogens by both experimental subjects. In confirmation of the results obtained by Weinfeld and Christman (2), the present work demonstrates that 60 to 80 per cent of this chromogenic material is 1-methyluric acid and the remainder 1,3-dimethyluric acid (Table II).

The analytical procedures were similar to those previously described. Eluates from paper chromatograms corresponding to those of uric acid (R_F 0.20), 1-methyluric acid (R_F 0.42), and 1,3-dimethyluric acid (R_F 0.54) gave absorption maxima at 285 mμ. The values given in Table II were calculated from the optical densities observed at this wave length. A small amount of material (10 to 15 mg. per 24 hours) with a spectra characteristic of the uric acids was found in the eluate from the chromatogram

at R_F 0.63 (0.58 to 0.68). The only methyluric acid found at this R_F range is 1,7-dimethyluric acid (R_F 0.60). Johnson (9) has reported the presence of 1,7-dimethyluric acid in the urine of individuals on a regular diet, which included coffee and tea. No evidence was found for the presence of 3-methyluric acid, which has an R_F value of 0.32.

If demethylation of caffeine without oxidation occurred, six mono- and dimethylxanthines, as well as unchanged caffeine, might be present in the xanthine eluate. Unchanged caffeine (R_F 0.78) was well separated from the other xanthines by paper chromatography. The material eluted from this area of the chromatogram (R_F 0.73 to 0.84) was characteristic of caffeine since it was not precipitated as a silver salt and showed no shift in absorption peak in changing from acid to alkaline solution. After the ingestion of 1 gm. of caffeine, 11 and 12 mg. were excreted unchanged by subjects H. C. and F. M., respectively.

The material which was eluted from the chromatogram with an R_F of 0.66 (0.59 to 0.73) had absorption peaks in acid at 262 mμ and in alkaline solution at 283 mμ. This shift is typical of both 7-methyl- and 1,7-dimethylxanthine but the R_F value is that of 1,7-dimethylxanthine (0.66 rather than of 7-methylxanthine (0.43). After silver precipitation at pH 1.0, the absorption curve of the reconstituted xanthine was still typical of 1,7-dimethylxanthine with some evidence, however, for the presence of a small amount of 1-methylxanthine (R_F 0.55). Upon adjustment of the supernatant fluid to a pH of 5.0 to 6.0, a second silver precipitate was obtained. The reconstituted xanthine from this precipitate had an absorption spectrum in alkaline solution with maxima at 240 and 272 mμ, typical of 1-methylxanthine (242 and 275 mμ). The small amount of 1,7-dimethylxanthine that was not precipitated at a pH of 1.0 to 2.0 would be included in this fraction.

Approximately one-third of the ultraviolet-absorbing material of the eluate with an R_F of 0.53 (0.47 to 0.59) was precipitated as the silver salt at pH 1.0 and 90 per cent of the remainder at pH 5.0 to 6.0. Similar percentages of 1-methylxanthine added to a control urine were precipitated as silver salts at these pH values. The spectra of the xanthine released from these silver precipitates had peaks in alkaline solution at 240 and 277 characteristic of 1-methylxanthine.

The material eluted from the chromatogram with an R_F of 0.42 (0.37 0.47) appeared to be 7-methylxanthine for several reasons. (1) The R_F value of 7-methylxanthine in this solvent is 0.43. (2) The absorption peak of the material in acid solution is at 269 mμ, which is similar to that 7-methylxanthine. Moreover, the 18 mμ shift in peak in alkaline solution to 287 mμ is characteristic of 7-methylxanthine. (3) Data in Table I indicate that, at pH 1.0, 92 per cent of 7-methylxanthine is precipitated as the

silver salt. 90 per cent of the ultraviolet-absorbing material in the eluate with an R_F of 0.42 was also precipitated at pH 1.0.

The values given in Table II for the amounts of the various catabolites of caffeine excreted per 48 hours were calculated from the optical densities of the reconstituted xanthines after silver precipitation. Since caffeine is not precipitated as a silver salt, the values for caffeine were obtained from the optical densities of the eluates from the paper chromatograms.

DISCUSSION

Since the development of the enzymatic uricase method for the determination of uric acid, all studies have indicated that there is no appreciable

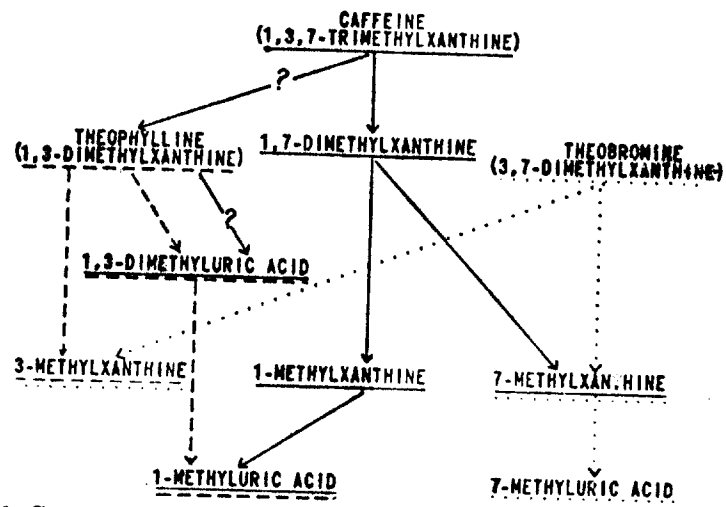

FIG. 1. Compounds excreted by man after the ingestion of caffeine (solid line) theophylline (dashed line), and theobromine (dotted line).

increase in true uric acid excretion upon the ingestion of caffeine, theophylline, or theobromine. Increases in the excretion of material which gives color with uric acid reagents after the ingestion of caffeine and theophylline can be attributed to the presence of the chromogenic methyluric acids.

In Fig. 1, the probable metabolic pathways of caffeine, theophylline, and theobromine in man are presented. After the ingestion of 1 gm. of theobromine, the major excretory products found in the urine of two subjects expressed as per cent of dose were 7-methylxanthine (28 to 30), 1-methylxanthine (14 to 21), and unchanged theobromine (11 to 12). A small amount of 7-methyluric acid (3 to 4 per cent) was also excreted. Since more 7- than 3-methylxanthine was excreted, it would appear that demethylation occurs more readily at the 3 than at the 7 position. The

7-methyluric acid may be formed by the oxidation of 7-methylxanthine or by demethylation of 3,7-dimethyluric acid formed by the direct oxidation of theobromine.

Theophylline (1,3-dimethylxanthine) differs from theobromine in that both of the methyl groups are on the pyrimidine ring, leaving the imidazole ring unsubstituted. The main excretory products after theophylline ingestion were 1,3-dimethyluric acid (35 per cent), 1-methyluric acid (19 per cent), 3-methylxanthine (13 per cent), and unchanged theophylline (10 per cent). The oxidation of theophylline without demethylation appears to be the major metabolic pathway. In addition a portion of the theophylline was demethylated at position 1 to give 3-methylxanthine. Two possible pathways for the formation of 1-methyluric acid are as follows: (1) demethylation of 1,3-dimethyluric acid or (2) direct oxidation of 1-methylxanthine. Although this latter compound has not been detected as one of the excretory products of theophylline metabolism, it may be an intermediate.

It would appear that theobromine and theophylline follow somewhat different pathways of metabolism. The major products of theophylline metabolism in man are methyluric acids, and the major products of theobromine metabolism are methylxanthines. Direct oxidation of theophylline must occur with relative ease to produce large amounts of 1,3-dimethyluric acid. On the other hand, no 3,7-dimethyluric acid was found upon the ingestion of theobromine, although small amounts may have been formed and further metabolized. Apparently a methyl substitution at position 7 hinders oxidation at the adjacent C atom (position 8). That oxidation is not completely prevented is shown by the excretion of small amounts of 7-methyluric acid after the ingestion of theobromine.

Excretion of 1-methyluric acid and 1-methylxanthine accounted for approximately 46 per cent of the ingested caffeine. In addition to these two products, 1,7-dimethylxanthine, 7-methylxanthine, 1,3-dimethyluric acid, and unchanged caffeine were present. All of these products, except the 1,3-dimethyluric acid, may be accounted for by a direct pathway of demethylation and oxidation via 1,7-dimethylxanthine. Weinfeld (10) has pointed out that, if the primary step is a demethylation in the 7 position, caffeine would be converted to theophylline which would then be metabolized to yield the series of compounds characteristic of this dimethylxanthine. On the other hand an initial demethylation at position 1 would yield theobromine, which would be further metabolized to give its characteristic products.

Since the pattern of the excretory products after caffeine ingestion is different from that of theophylline or theobromine, other metabolic pathways must be considered. 1,7-Dimethylxanthine, which is excreted in small amounts, may be the intermediate in the formation of both the 1-

methyl and 7-methylxanthine. Oxidation of 1-methylxanthine would give 1-methyluric acid. The 1,3-dimethyluric acid could arise from the demethylation of 1,3,7-trimethyluric acid formed by direct oxidation, even though none of this compound is excreted. It is more probable that this dimethyluric acid comes from the oxidation of 1,3-dimethylxanthine. Although this latter compound is not excreted after caffeine ingestion, it may be formed at a rate which permits its complete oxidation.

The present work indicates that demethylation may occur at either the 1, 3, or 7 position. In man, the order of increasing stability of these methyl groups is 3, 7, and 1. This is consistent with the chemical lability of these positions as determined by Cavalieri *et al.* (11), who found that the most readily dissociated hydrogen of xanthine is at position 3 and the least acid hydrogen is at position 1. That demethylation of caffeine, theobromine, and theophylline in man does not go beyond the monomethylxanthines is supported by two lines of evidence: (1) no accumulation of xanthine in the urine was observed in the chromatographic studies; and (2) insignificant, if any, increases in uric acid excretion.

SUMMARY

After suitable control periods on diets low in methylated purines, two human subjects received 1 gm. doses of theobromine, theophylline, and caffeine. 62 per cent of the theobromine, 77 per cent of the theophylline, and 66 per cent of the caffeine were excreted in the form of methylxanthines and methyluric acids during the subsequent 48 hours. The major part of the theobromine was excreted as methylxanthines. In contrast, the methyluric acids were the predominant excretory products of theophylline. After caffeine administration, approximately equal amounts of the methylxanthines and methyluric acids were present in the urine.

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ON THE EFFECT OF CAFFEINE ON SPINAL REFLEXES*

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While the effect of caffeine on the cerebral cortex has been studied in considerable detail, little work has been done on its effect on the spinal cord. Ostashkov (1953, 1955a, 1955b, 1956) has shown in acute experiments on spinal frogs and decerebrate cats that it promotes the irradiation of

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excitation and intensifies Sechenov inhibition. In moderate doses it leads to more rapid restoration of the reflex excitability of the spinal cord, but in large doses, to its exhaustion. According to the findings of Val'dman (1950a, 1950b), caffeine produces no material change in the value or latent period of the patellar reflex in intact rabbits. Kochetova (1955) failed to observe any regular changes in the patellar reflexes of human subjects as a result of caffeine administration, whereas Amitin (1955) and Dubenko (1955) observed definite change in the reflex which, in their opinion, was explained by negative induction from the excited cortex. Iakovleva (1941, 1953), working with dogs, and Akhmarov (1956), working with human subjects, noted reduction of the motor chronaxie in the muscles of the hind (lower) extremity from caffeine and ascribed this to a subordination effect from the excited cortex.

Our object was to make a detailed study of the effect of caffeine on the reflex activity of the spinal cord.

METHOD

Three successive series of chronic experiments were carried out. We investigated the effect of different doses of caffeine on several physiologically and morphologically different reflex arcs, namely, the patellar reflex, its inhibition, and the chronaxie of the flexor reflex in the hind legs of intact dogs, dogs with the spinal cord transected and dogs with both the spinal cord transected and partial bilateral sympathectomy. Additionally, the effect of caffeine on the crossed extensor reflex was studied in the last two series of experiments.

Complete transection of the spinal cord was effected between the 10th and 11th thoracic vertebrae. The sympathectomy was carried out by the method described by Afanas'ev (1956) in two stages, one month after the cord transection on one side and 15–20 days after the last operation on the other side. The thoracic sympathetic chains (from the 6th to the 11th sympathetic ganglion) were removed on both sides. As a preliminary measure, the dogs were trained to lie on their back in a special stand. Experiments were carried out daily.

As in the studies of Kunstman (1928), Iankovskaia (1940), Fedotov (1951) and Bobrova (1959), the patellar reflex was elicited by blows with a percussion hammer on the tendon of the quadriceps muscle of the left leg in time with the metronome beating at rates of 360, 240, 120 and 60 beats/min with 5 min intervals between rates.

Reciprocal inhibition of the patellar reflex was achieved by unipolar stimulation of the skin on the dorsum of the foot of the leg being examined. Stimulation lasting 5 sec was delivered from an induction coil (without interrupter) during the rhythmical production of the patellar reflex. The source of current was the town supply (50 periods/sec), reduced by means of a transformer to 5 V. A similar method was used for inhibition of the patellar reflex in the investigations of Fedotov (1951) and Bobrova (1959).

The reflex flexor chronaxie in the hind limbs was determined by means of an apparatus on the lines of Bourguignon's circuit (1929). The active silver electrode (diameter 1·5 mm) was placed on the dorsum of the foot (at the base of the toes) and the inactive electrode was on the skin of the thorax, which was thoroughly shaved and moistened with saline. The criterion of excitation was slight but definite

flexion of the toes. Chronaxie was determined twice (2–3 measurements each) at 10 min intervals in each leg. The coefficient used for conversion of microfarads to msec was 4.

The crossed extensor reflex was recorded jointly with the patellar reflex (special tests), but in some of the experiments it was recorded separately.

Caffeine (caffeinum purum) in doses of 0·0035–0·008, 0·02–0·035, 0·04–0·08 g/kg (hereafter these doses will be termed "small", "medium" and "large") was administered internally in a milk-water mixture 30 min before the commencement of an experiment. As a control, a mixture of milk and water without caffeine was also given 30 min before an experiment.

RESULTS

Effect of caffeine on the patellar reflex and its inhibition. (Table 1). Experiments totalling 552, 64 with caffeine, were carried out on five intact dogs. Reduction of the patellar reflex (Fig. 1) was noted in 37 of the 64 caffeine experiments, phasic changes were seen in 8, increase in 6 and in 13 experiments no material changes were detected (8 of these were experiments with small doses of caffeine).

TABLE 1. EFFECT OF CAFFEINE ON THE KNEE REFLEX AND ITS INHIBITION

Dogs	Amplitude				Inhibition			
	Reduction	Phasic changes	Increase	No change	Reduction	Phasic changes	Increase	No change
Intact	37	8	6	13	39	9	15	1
Spinal	22	7	10	1	28	—	9	3
Spinal and sympathectomized	4	14	—	3	8	12	1	—
Total	63	29	16	17	75	21	25	4

Inhibition of the patellar reflex was increased (Fig. 1) in a majority of cases (in 39 of 64 experiments), enfeeblement of inhibition or phasic changes being seen less frequently. The changes seen with medium and large doses of caffeine were more pronounced and more prolonged (3–5 days). Enfeeblement of patellar reflex inhibition was most frequently encountered with small doses of caffeine (7 of 21 experiments) and less frequently with the medium and large doses. If, however, account is taken of the fact that, in the case of phasic changes, intensification of the inhibitory effect was generally noted in the first one or two days after the administration of caffeine, the number of cases of intensified inhibition would be 48 and not 39 in the 64 experiments. Generally, when the inhibitory effect was intensified the amplitude of

the patellar reflex was reduced and, conversely, it was increased when inhibition was reduced.

Two hundred and fifty-four experiments, 40 with caffeine, were carried out on six dogs with complete spinal cord transection. There was reduction of the patellar reflex in 22 of 40 experiments, phasic changes in 7, and increase of the reflex in 10 experiments. In the phasic changes reduction of the patellar reflex was observed on the day of caffeine administration and sometimes also on the second day after which it was replaced by increase. Increase of the patellar reflex occurred mainly with small doses of caffeine (in 7 of 18 experiments).

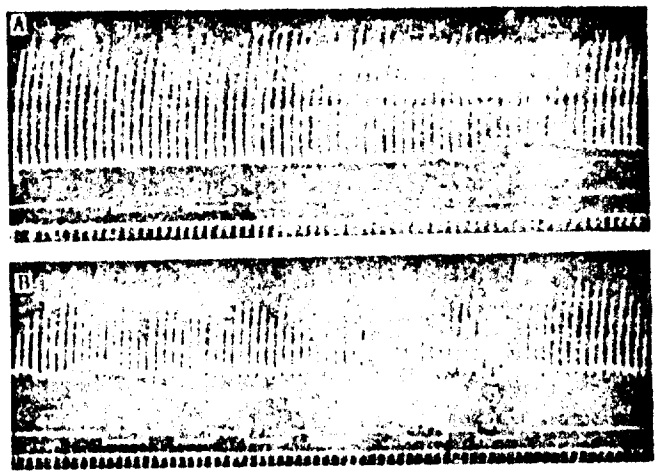

FIG. 1. Reduction of the patellar reflex and increase of its inhibition after the administration of 0·007 g/kg caffeine. Dog Fibra (intact). (A) Before administration. (B) On day of caffeine administration. From above down: recording of the patellar reflex (at a rate of 60); stimulation of the ipsilateral paw; time (sec). The numbers indicate the distances between the coils of the induction apparatus.

Inhibition of the patellar reflex was more frequently increased by caffeine (in 28 of 40 experiments), and weakening was seen less frequently (in 9 of 40 experiments). Weakening of the inhibition occurred more frequently with small doses of caffeine (6 of 9 experiments).

One hundred and fifty-seven experiments, 21 with caffeine, were carried out on the three sympathectomized spinal dogs. Phasic changes in the patellar reflex (Fig. 2) were produced by caffeine in 14 of 21 experiments, the reflex was reduced in 4 and was unchanged in 3 experiments.

In a majority of instances the inhibition of the patellar reflex likewise suffered phasic changes (enfeeblement following intensification) or was increased: there were phasic changes in inhibition (Fig. 2) in 12 of 21

experiments, the inhibition was increased in 8 and was reduced in one experiment. Increase of the inhibitory effect was seen more frequently with small doses of caffeine (6 of 7 experiments) and the phasic changes with medium and large doses. The inhibitory effect was generally increased in the first phase and reduced in the second. The first phase, intensification of inhibition, was seen on the day of caffeine administration and sometimes on the second day, being replaced thereafter by the second phase, the phase of enfeeblement.

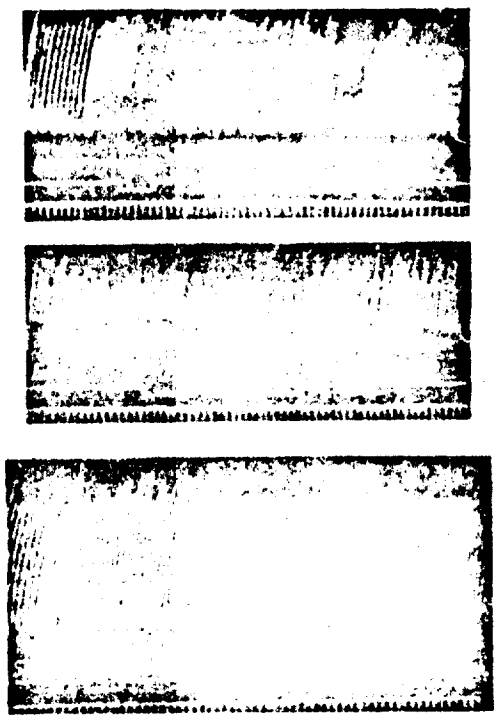

FIG. 2. Phasic changes in the patellar reflex and in its inhibition after the administration of 0·02 g/kg caffeine. Dog Groznaia (sympathectomized spinal dog). (A) Before administration. (B) On the day of administration. (C) On the second day after administration. Notation as in Fig. 1.

The patellar reflex was thus reduced and its inhibition increased in a majority of instances in intact and spinal dogs. These indicators were often subject to phasic changes in the sympathectomized spinal dogs.

Effect of caffeine on the chronaxie of the flexor reflex. Seventy-five experiments with caffeine were carried out on 6 intact dogs. In most instances the administration of caffeine was followed by reduction of the chronaxie of the flexor reflex, less frequently by asymmetrical phasic changes in or increase of chronaxie (Table 2). Chronaxie became reduced

TABLE 2. EFFECT OF CAFFEINE ON THE CHRONAXIE OF THE FLEXOR REFLEX AND ON THE CROSSED EXTENSOR REFLEX

Dogs	Chronaxie					Crossed extensor reflex			
	Reduction	Phasic change	Asymmetric change	Increase	No change	Increase	Reduction	Phasic change	No change
Intact	39	19	7	6	4	—	—	—	—
Spinal	13	1	4	6	2	11	5	7	1
Spinal and sympathectomized	8	16	—	—	1	2	2	17	—
Total	60	36	11	12	7	13	7	24	1

in 39 of 75 experiments (Fig. 3). The value of chronaxie was reduced by 30–60 per cent or more after the administration of caffeine. The reduction could be observed for 1–2 days with small doses and for 3–5 days with medium and large doses of caffeine. There were phasic changes in 19 of

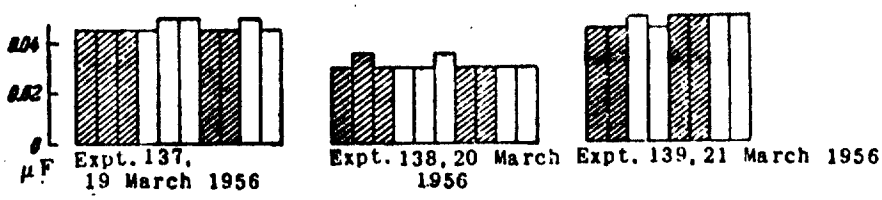

FIG. 3. Reduction of chronaxie after administration of 0·007 g/kg caffeine. Dog Ingus (intact). Shaded columns: chronaxie in the left leg. Blank columns: chronaxie in the right leg. The chronaxie values are given in microfarads.

the 75 experiments. The first phase of reduction was always seen on the day of caffeine administration or on the following day, being then replaced by the second phase of increase. Asymmetrical change, i.e. reduction of chronaxie in one paw and increase in the other, was seen in 7 experiments. Increase of chronaxie occurred in 6 of the 75 experiments and was observed in only two dogs.

Twenty-six caffeine experiments were carried out on the three spinal dogs. In most cases reflex chronaxie was reduced by the action of caffeine. In the case of two dogs chronaxie was always reduced after caffeine, but asymmetrical changes were sometimes seen on the second or third day after the administration. Chronaxie was generally increased by caffeine in the third dog.

Twenty-five experiments with caffeine were carried out on three sympathectomized spinal dogs. There were phasic changes in the chronaxie

of the flexor reflex in 16 of 25 experiments (Fig. 4) and reduction of chronaxie was seen in 8 experiments. Reduction of chronaxie was mainly observed with small doses of caffeine (in 8 of 10 experiments). With medium and large doses phasic changes were generally observed, chronaxie being reduced on the day of caffeine administration and increased on the second or third day thereafter.

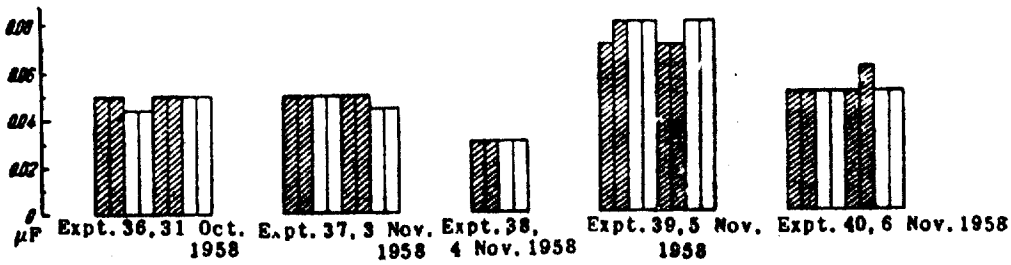

FIG. 4. Phasic changes in the chronaxie of the flexor reflex after administration of 0·045 g/kg caffeine. Dog Astra (sympathectomized spinal dog). Notation as in Fig. 3.

Effect of caffeine on the crossed extensor reflex. Examination of the effect of caffeine on the crossed extensor reflex was carried out parallel with the recording of the patellar reflex (see Table 2).

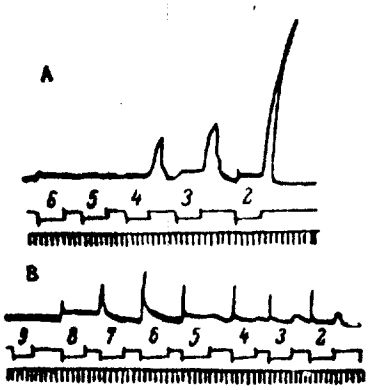

FIG. 5. Equilibrated phase of parabiosis after the administration of 0·06 g/kg caffeine. Dog Chernaia (sympathectomized spinal dog). (A) Before administration. (B) On the day of caffeine administration. From above down: recording of the crossed extensor reflex; stimulation delivered to produce this reflex; time (sec). The numbers indicate the distance between the coils of the induction apparatus.

There was lowering of the thresholds for the crossed extensor reflex lasting four days after medium and large doses of caffeine and 1–2 days after small doses in 11 of the 24 experiments carried out on the three spinal dogs. Phasic changes in the excitation thresholds and amplitude

of the crossed extensor reflex were observed in 7 of the 24 experiments. The first phase took the form of lowering of thresholds and was seen on the day of administration or the next day, after which the thresholds rose; this state continued for a further 1–2 days. Raising of the thresholds for the extensor reflex occurred in five experiments (of 24) and was seen mainly with small doses of caffeine.

Twenty-one caffeine experiments were carried out on the three sympathectomized spinal dogs. In most cases the crossed extensor reflex underwent diphasic changes (17 of 21 experiments). The first phase, lowering of the thresholds and increase in the amplitude of the reflex, was observed on the day of caffeine administration, and only in occasional cases did it extend to the second day. Depression (second phase) of the crossed extensor reflex usually developed on the second day but sometimes also with medium and large doses on the third or fourth day. There was marked depression of the reflex on the second or third day after the administration of medium and large doses of caffeine, and in some cases the reflex could not be produced at all. Parabiotic phases, equilibrated (Fig. 5) and paradoxical, sometimes developed after the administration of medium and large doses of caffeine.

DISCUSSION

The experiments on intact, spinal and sympathectomized spinal dogs showed that the spinal reflexes were altered by caffeine in all the series of experiments. As caffeine has an exciting effect on the cerebral cortex, it can be assumed that the changes noted in the intact dogs developed as a result of subordination effects from the cerebral cortex excited by caffeine. More particularly, the reduction of the patellar reflex in the intact dogs could be regarded as the result of negative induction. In our experiments reduction of the patellar reflex predominated not only in the intact dogs, but also in the spinal and sympathectomized spinal dogs (first phase), in which any possibility of effects from the higher divisions of the central nervous system on the caudal segment of the divided spinal cord was excluded. There is, therefore, reason to believe that caffeine acts directly on spinal centres. Why then should caffeine depress the patellar reflex and, at the same time, enhance the excitability of the centre for the flexor reflex? Apparently caffeine increases the excitability of the centre of the flexor reflex to a greater extent than the centre for the patellar reflex, and this possibly led to the reciprocal inhibition of the patellar reflex which developed during the rhythmical activity of the reflex. The intensification of the reciprocal inhibition of the patellar reflex when the latter was being elicited rhythmically is probably explained by the fact that coffeine promotes rapid irradiation of excitation

in the spinal cord (Ostashkov, 1955a). The excitation arising during the production of the patellar reflex irradiated rapidly through the spinal cord, particularly to the centre for the flexor reflex and thus increased its physiological predominance. Ukhtomskii and Vinogradov (1925) noted that the flexor reflex usually predominated over the extensor reflex in spinal frogs. Rozentsvaig (1937) likewise stated that the excitability of the flexor reflex centre was greater than that of the extensor reflex centre; this relationship persisted even in the presence of pathological changes in the central nervous system. Perli (1950, 1957) and Peimer and Perli (1950) drew attention to reduction of the patellar reflex and simultaneous development of potentials in the flexors and extensors in relation to blows on the patellar tendon, and they explained these phenomena by the facile irradiation of excitation and the existence of reciprocal relationships between the centres for the flexor and patellar reflexes.

The reduction of the chronaxie of the flexor reflex and the increase of the crossed extensor reflex indicated that caffeine increased the excitability of the centres for these reflexes, or in other words, there was evidence of the typical exciting effect of caffeine.

We are inclined to explain the predominantly phasic changes in the reflexes investigated in the spinal sympathectomized dogs by the fact that sympathectomy increases the sensitivity of the spinal cord to caffeine and that the previously excited centres for the flexor and crossed extensor reflexes sink more rapidly into a state of prohibitive inhibition, with the development of equilibrated and paradoxical stages, than is the case when there is no sympathectomy. Khanutina (1939), Stefantsov (1954) and others have also drawn attention to increase of sensitivity and disturbance of the balance between excitation and inhibition in the central nervous system in the presence of a lesion of the sympathetic division of the nervous system.

CONCLUSIONS

(1) Chronic experiments on intact, spinal and sympathectomized spinal dogs showed that caffeine produced profound shifts in the reflex activity of the spinal cord.

(2) Reduction of the patellar reflex, increase of its inhibition, shortening of the chronaxie of the flexor reflex and lowering of the thresholds and increase in the amplitude of the crossed etensor reflex were observed to result from caffeine administration in intact and spinal dogs in a majority of instances. Less frequently there was increase of the patellar reflex, reduction of its inhibition, increase of the chronaxie of the flexor reflex and raising of the thresholds and reduction in the size of the crossed extensor reflex. In the sympathectomized spinal dogs these indices generally underwent phasic changes.

(3) The intensity and duration of the caffeine effect on the spinal cord depended on the dose, the duration being 1–2 days with small doses and 3–5 days with medium and large doses.

(4) Caffeine had a direct effect on spinal cord centres.

Translated by R. CRAWFORD

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Effects of Caffeine on Human Sugar-Tolerance Curves.*

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There is considerable evidence that caffeine affects the blood sugar of animals. In dogs, a rise follows caffeine injection[1] amounting in some cases to 40-60% above normal[2] in both arterial and venous blood;[3] a double dose is necessary to produce hyperglycemia during avitaminosis.[4] During work,[5] the sugar tolerance curve rises more rapidly with caffeine than without and stays up longer.

However, caffeine produces no hyperglycemia in castrated female rabbits,[6] nor does it affect the glycogen contents of the heart or liver in rats and guinea pigs.[7]

No report could be found regarding the effect of caffeine on the blood sugar or sugar tolerance curve for man.

Eight sugar tolerance curves were determined on the same subject (M.D.) using the sugar method of Folin and Malmros.[8] The nutri-

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tional history was nearly the same in all cases. No food was taken after 5 p. m. the day before, the amount of sleep and activity was similar, and the determinations were made at the same time of day, 10 a. m.—1 p. m. Fingertip blood samples were taken every 10 minutes for the first 1½ hours after ingesting the dextrose. All sugar determinations were made in duplicate.

The data and results from 4 of the experiments are shown in Fig. 1.

These results indicate that large doses of caffeine depress the peak of the human sugar-tolerance curve and slightly delay the return to normal. Caffeine alone does not raise the blood sugar appreciably. The amounts commonly ingested in coffee and tea probably do not significantly affect either the human tolerance curve or the blood sugar levels.

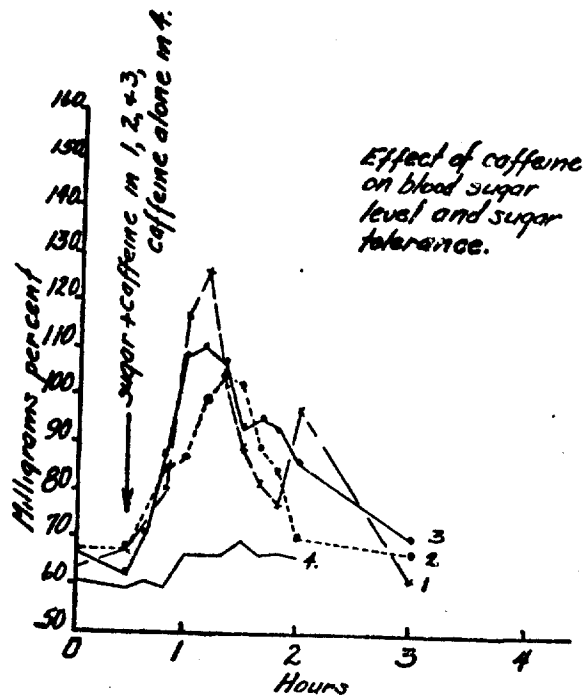

FIG. 1.

1. 50 g dextrose + 200 cc distilled water.
2. 50 g dextrose + 0.5 g caffeine + 200 cc distilled water.
3. 50 g dextrose + 200 cc coffee (9 g coffee/100 cc water).
4. 0.5 g caffeine + 200 cc distilled water.

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Visual threshold as a function of test area and caffeine administration*

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The threshold luminance of a small circle was studied as a function of its area (varied from 2.69 min to 26.87 min in radius) and for different dosages of caffeine (0, 1.5, and 3 grains). The zero caffeine condition shows the classical decrease in threshold with increased area. With caffeine administration, the slope of this threshold-area function remains unchanged, although the entire function shifts downward (threshold decreases equally for all areas) increasingly with an increasing amount of caffeine. The results combined with previous caffeine experiments suggest a theoretical interpretation that caffeine causes the "on" visual pathways to become more sensitive to light.

The threshold luminance necessary to see a small single visual field decreases as the area of the field increases (Graham, Brown, & Mote, 1939; Diamond, 1962). This is an illustration of single-field brightness enhancement as suggested by Diamond (1960). That is, the sensitivity to light is enhanced as the area of a field is increased.

Heinemann (1955) found the brightness of a test field of constant luminance increased (was enhanced) when he surrounded the test field with an annulus of very low luminance. This is an illustration of multiple-field enhancement. Kleman et al (1961) repeated Heinemann's results and further found that administration of three grains of caffeine to the Ss resulted in the apparent disappearance of the enhancement.

It is this elimination of enhancement by caffeine that is the interest of the present investigation. The question is, will caffeine eliminate or reduce enhancement in a single field as well as in the Kleman multiple-field visual situation?

The present experiment, designed to test this question, measures the threshold luminance of a test field as its area is increased from 2.69 min to 26.87 min in radius. This is done for three dosages of caffeine, 0, 1.5, and 3.0 grains. (Kleman used 3 grains.) It is expected that threshold luminance will decrease as a function of test area, illustrating enhancement. On the other hand, if caffeine is effective in reducing enhancement, the slope of the threshold decrease should lessen with increased caffeine.

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APPARATUS

The apparatus, presented in Fig. 1, consists of a light path to S's right eye. A 150-W frosted tungsten-filament bulb operated at 115 V ac constitutes the light source. The light, diffused by flashed opal glass, passes through 1 of 10 test circles (of different areas), a crossed Polaroid filter (for continuous luminance control), and fixed-density Wratten filters (for discrete luminance control). Next, the light is made parallel by a 4-diopter lens, and finally it passes through a 3-mm artificial pupil to the eye of S, who is seated in a dark room. The artificial pupil is mounted in an optician's trial frame fixed to S's head in order to reduce variability of brightness judgments due to head movements (Diamond, 1955).

A fixation point, a 3-min point of light, is located 43 min nasally from the center of the test field. Its source is a 50-W tungsten filament projection bulb operated at 115 V ac. Its point of introduction to the threshold light path between the variable and fixed Polaroid (see Fig. 1) is designed to permit constant fixation point luminance with variation of the variable Polaroid and, therefore, test luminance. The S is instructed to fixate this 3-min point of light and report the presence or absence of the test field as E manipulates the variable Polaroid. Since the visual angle between the fixation point and the outer edge of the test field does not exceed 70 min, retinal stimulation can be considered essentially foveal.

Test-field areas are varied in the following manner: 10 circular patterns, increasing in diameter in 0.0156-in. steps from 0.0156 in. to 0.1560 in., are cut in a thin metal disk that can be rotated and set so that any one pattern will fall directly in front of the diffuse light source in the optical path.

In addition to the apparatus elements shown in Fig. 1, the optical pathway also includes a Dove prism and a circular prism. However, these elements are nonfunctional in the present study.

PROCEDURE

Threshold luminance was explored as a function of the 10 test-field areas, their radii ranging from 2.69 min to 26.87 min in visual angle, under the following three experimental conditions: (1) The first consisted of oral administration of a placebo capsule containing 5 grains of calcium lactate; (2) the second consisted of administration of a capsule containing 1.5 grains of caffeine; and (3) the third consisted of administration of a capsule containing 3.0 grains of caffeine.

Experimental sessions were the same for each S for each experimental day. Fifteen minutes before entering the darkroom, S was given the placebo. At the end of this period, S entered the darkroom, adjusted the optician's trial frame, and then dark adapted for 15 min. Next, the S's range of thresholds was determined through the E's concurrent manipulation of the Wratten filters and variable Polaroid, and the S reported on the presence or absence of the test field while focusing on the fixation point. Once the range of thresholds for a particular session was established, all subsequent luminance variations could be accomplished with a variable Polaroid alone. Threshold measurements were then

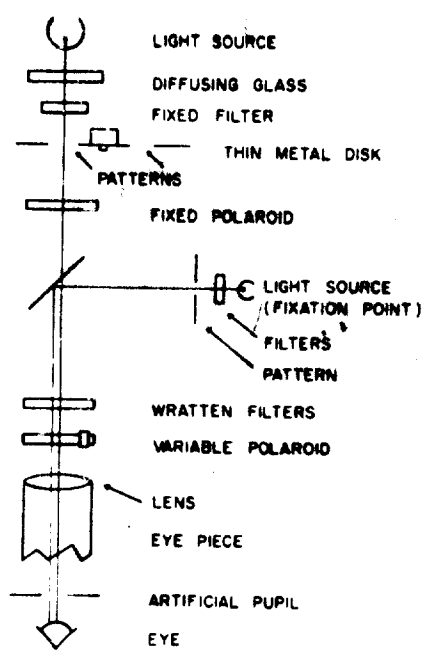

Fig. 1. Apparatus.

Table 1
Threshold Luminance as a Function of Test Radius for Caffeine and Placebo Conditions (in Log mL). (Data are for Ss JN, SI, and TH.)

	Drug Condition															
	1.5 Grains								3.0 Grains							
	Placebo				Caffeine				Placebo				Caffeine			
Test Radius (Minutes)	JN	SI	TH	Avg.	JN	SI	TH	Avg.	JN	SI	TH	Avg.	JN	SI	TH	Avg.
2.69	−2.27	−1.86	−2.00	−2.04	−2.26	−1.90	−2.22	−2.13	−2.36	−1.13	−2.23	−1.91	−2.72	−1.26	−2.33	−2.10
5.38	−2.37	−1.92	−2.10	−2.13	−2.34	−2.02	−2.30	−2.22	−2.53	−1.32	−2.39	−2.08	−2.78	−1.37	−2.47	−2.21
8.07	−2.47	−2.06	−2.21	−2.25	−2.43	−2.26	−2.40	−2.36	−2.59	−1.47	−2.57	−2.21	−2.83	−1.57	−2.57	−2.32
10.76	−2.46	−2.15	−2.31	−2.31	−2.56	−2.26	−2.50	−2.44	−2.63	−1.54	−2.58	−2.28	−2.90	−1.64	−2.72	−2.42
13.45	−2.60	−2.24	−2.48	−2.44	−2.61	−2.32	−2.58	−2.50	−2.74	−1.62	−2.79	−2.38	−3.05	−1.80	−2.82	−2.56
16.14	−2.64	−2.32	−2.54	−2.50	−2.71	−2.40	−2.68	−2.60	−2.85	−1.75	−2.91	−2.50	−3.16	−1.93	−2.94	−2.68
18.83	−2.74	−2.46	−2.55	−2.58	−2.74	−2.49	−2.73	−2.65	−2.92	−1.87	−2.98	−2.59	−3.22	−2.01	−2.96	−2.73
21.52	−2.80	−2.50	−2.66	−2.65	−2.79	−2.51	−2.84	−2.71	−3.06	−1.93	−3.00	−2.66	−3.26	−2.09	−3.06	−2.80
24.21	−2.90	−2.64	−2.74	−2.76	−2.82	−2.65	−2.88	−2.78	−3.20	−1.98	−3.12	−2.77	−3.40	−2.11	−3.14	−2.88
26.87	−2.89	−2.61	−2.77	−2.76	−2.92	−2.72	−2.86	−2.83	−3.26	−2.00	−3.13	−2.80	−3.44	−2.18	−3.14	−2.92

taken for the 10 test-field areas, the order being from smallest to largest.

At the conclusion of the placebo condition, S left the darkroom for a 1-h rest. Thirty minutes before reentering the darkroom, S was given either a 1.5-grain or a 3.0-grain capsule of caffeine, depending on the caffeine level to be tested during that session. The procedure described above under the placebo condition was then repeated. The order of presentation of the 1.5-grain condition and the 3.0-grain condition was randomized.

Two females, JN and SI, and one male, TH, served as Ss. All Ss were fully trained in the adjustment of the optician's trial frames and in focusing on the fixation point while making judgments. In an exploratory study, JN and TH were tested for placebo effects with the results showing no significant differences between threshold judgments under placebo vs nonplacebo conditions. Although the possibility of counterbalancing the three conditions was considered, it was decided not practicable, since caffeine has been found to affect visual behavior for as long as 6 h following administration (Kleman, Diamond, & Smith, 1961).

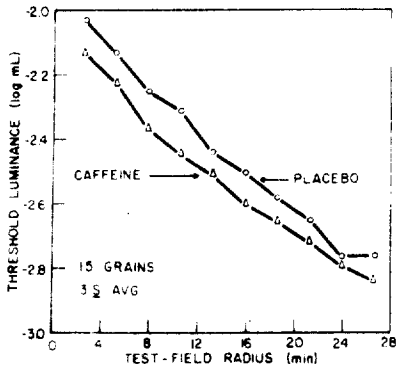

Fig. 2. Threshold in log mL as a function of test-field radius for 1½ grains of caffeine vs no caffeine (placebo condition).

During the above procedure, the psychophysical method of limits was used as described by Stevens (1951). From his position outside the darkroom, E slowly and evenly varied the luminance of the test field by moving the variable Polaroid filter in the optical pathway in a counterbalanced order of ascending and descending series. As the E slowly increased the luminance of the test field, S was instructed to inform the E when the entire test field with its boundaries appeared. Conversely, as the E slowly decreased the luminance of the test field, the S was instructed to inform the E when the boundaries of the test field became indistinct. The mean of three such pairs of ascending-descending judgments was taken as a measure of the test-field threshold luminance.

RESULTS

The data are presented in Table 1. The means of threshold luminances (log mL) are given separately for the three Ss for different days and for 10 test-field areas in addition to the combined means of the Ss. The latter values are graphed in Figs. 2 and 3.

The placebo condition curves show the enhancement phenomenon found by Graham, Brown, & Mote (1939) and Diamond (1962): i.e., as test-field area increases, test-field threshold decreases. With caffeine administration, furthermore, there is a decrease (from the placebo values) in the entire threshold function. This overall decrease, moreover, grows more pronounced as caffeine dosage is increased from 1.5 to 3.0 grains. There seems to be no systematic change, however, in the slope of the threshold-area curve as caffeine dosage is increased from 0 to 3 grains.

DISCUSSION

Since the results show no systematic change in the slope of the threshold-area curve with caffeine dosage increase, it seems reasonable to conclude that caffeine does not affect single-field enhancement, at least for the dosage (3 grains) that affects multiple-field enhancement.

Threshold does decrease systematically, however, over the entire threshold-area curve with increased caffeine dosage. The decrease itself is consistent with the results of Ditchburn & Power Steele (1941). This result raises an interesting theoretical possibility with respect to underlying physiological events.

Kleman et al (1961), as mentioned above, found that enhancement decreases with caffeine administration in the multiple-field situation (a test circle surrounded by an annulus). One speculation is that "on" fibers in the retina (see Hartline, 1940) become more sensitive with caffeine administration and, as a result, depression of the test field by the increasingly active inducing-field "on" fibers increases, and thus reduces or cancels out enhancement. Unfortunately, since his measure was a binocular match, Kleman's data does not allow detection of changes in the absolute sensitivity of the eyes, since both eyes of the Ss presumably were affected equally by the caffeine.

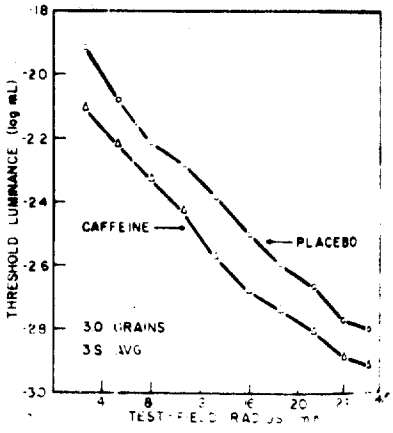

Fig. 3. Threshold in log mL as a function of test-field radius for three grains of caffeine vs no caffeine (placebo condition)

On the other hand, the threshold measurement in the present experiment does indicate absolute sensitivity. And since the data show that absolute sensitivity increases (threshold energy decreases) with caffeine administration, this fact lends support to the speculation that retinal "on" fibers become more sensitive with caffeine.

Why then was enhancement also not affected in the present threshold experiment, as it was in Kleman's brightness match experiment?. One explanation may be in the probable constancy of "on" fiber activity for a threshold response. That is, regardless of the amount of stimulus or whether or not caffeine has been administered, we might assume that the minimal amount of physiological activity necessary to produce the threshold response remains constant, since the threshold sensation by definition and by instruction is presumed constant. For threshold measurement in the present experiment, therefore, although the "on" fibers might become more sensitive, the amount of "on" activity remains the same regardless of the administration of caffeine. If "on" activity, therefore, is in fact constant, we would predict no change in enhancement. (In Kleman's experiment, "on" activity might be assumed to have increased with caffeine administration although, as pointed out above, his binocular match procedure did not provide a measure of such a change.)

One further consideration involves the role of the "off" fiber in the retina. Diamond (1960) suggests that both single- and multiple-field enhancement can be explained in terms of an inhibition of "off" retinal activity resulting in a disinhibition (see Hartline & Ratliff, 1959) of test brightness. In the single-field situation, as the test area increases, its light turns off more and more "off" fibers that were initially depressing the test-field's "on" activity (see Granit, 1955, for a discussion of "on-off" antagonism). For Kleman's multiple-field enhancement, the introduction of a low luminance surround theoretically eliminates "off" activity in the area surrounding the test field and again the disinhibited test brightness increases.

Since enhancement did not change in the present experiment, then, according to the above theoretical account, "off" fiber activity probably was not affected by caffeine administration, even though "on" fiber sensitivity was. In this connection, it is interesting to note that a number of investigators suggest differences between "off" and "on" retinal systems (MacNichols & Love, 1960; Ratliff, 1965), whereas others suggest that the "off" response might be a "rebound" (Florey, 1961) or a "net summation" (Kennedy, 1963) of the "on" responses. The results of the present study, interpreted in the light of Diamond's theoretical account (1960), would tend to support the idea of some functional difference between "on" and "off" systems and that this difference, whatever it might be, shows up as a differential response to caffeine, the "on" system being made more sensitive to stimuli and the "off" system not significantly affected.

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CAFFEINE-WITHDRAWAL HEADACHE*

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THE production of the experimental headache for investigative purposes was initiated by the observations of Loevenhart[2] and his co-workers in 1927, who found that nitrite headaches were accompanied by a dilatation of the meningeal blood vessels. Pickering[3] in 1933 studied headaches produced by the intravenous injection of small amounts of histamine. These studies were enlarged by Clark et al.[4] in 1934 and by Wolff et al.[5] in 1940, who demonstrated that this experimental headache was accompanied by an increased excursion of the cerebral blood vessels. Carbon monoxide headaches have been studied in a few individuals (Cobb et al., 1924),[6] but the toxic nature of carbon monoxide does not lend itself to human experimentation. The experimental headaches studied thus far do not simulate any of the so-called common headaches in mode of onset or duration. The histamine headache, however, is by far the most constant and may be produced in almost all subjects.

Because many people ascribe an occasional headache to lack of their morning coffee, and because considerable "letdown" must result if this stimulant drug is withdrawn from habituated individuals, an attempt to produce and study such a headache was undertaken. In a recent survey of one hundred and twenty-eight migraine patients, twenty-five stated that lack of their usual coffee intake would result in headache. Five patients volunteered the information that the headache was not of the migraine type. In this study we have attempted to produce headache by administration of caffeine over a suitable period, usually a week, and then abruptly withdrawing the drug.

Data obtained in this laboratory[7] have indicated that there is a relative hemoconcentration accompanying migraine. Hence, it was thought advisable to determine total proteins and hematocrits during the course of the experiments. Several investigators[8-11] have reported that the serum calcium is decreased in migraine patients. Therefore, it was thought possible that electrolyte changes in the blood might also be correlated with any period of headache produced by caffeine withdrawal.

EXPERIMENTAL

Caffeine, 0.120 Gm. capsules (gr. ii) as the sodium benzoate or citrate salt, was administered to normal subjects in an increasing daily dosage up to 0.78 Gm. (gr. xii), usually given in the morning. Twenty male and two female subjects (graduate and medical students) were used. Their ages varied from

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†Lieutenant (M.C.), U.S.N.R.

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20 to 33 years. They were not hospitalized, but they were observed and had their symptoms recorded daily. No regulation of food intake was attempted. Only three of the twenty subjects were normally total abstainers from caffeine-containing beverages. In order to add to our knowledge of the migraine syndrome, five of the subjects were particularly chosen because they had had typical periodic migraine headaches for at least two years. The subjects were given a series of ten or more dated envelopes, each containing the same number of capsules, placebo capsules and caffeine capsules being mixed in such a way that the subject would start with a small dose of caffeine the first day, but increasing daily so that by the sixth or seventh day the subject would be receiving 10 to 12 grains of caffeine per day. On the seventh or eighth day placebo capsules were substituted without the subject's knowledge, so that no caffeine was received thereafter. During the experiment the subject abstained from caffeine beverages, and in most cases the subjects had no caffeine beverages for the 24 to 48 hours preceding the beginning of administration. The experiment was postponed if the subject had a headache, and this occurred in five instances when habituated subjects discontinued their customary daily coffee intake.

Five blood samples were taken during each experiment. An initial control sample was taken before administration of caffeine was started, a second at the height of the caffeine stimulation, a third on the day before withdrawal, a fourth at the height of the headache, and a fifth on the following day when the subject was free of headache. In tabulating the results, the first three samples on each subject have been averaged, since they were found to be uniformly the same. At the time the blood samples were taken, blood pressure and pulse were recorded after the subject had been supine for five minutes. All control observations and samples were taken between 4 P.M. and 8 P.M. after it was found that this was usually the time of maximum headache.

Serum calcium, serum inorganic phosphorus, serum potassium, serum protein, serum and blood specific gravity, and hematocrits were determined as in previous studies.[12] The calcium samples were stored at a stable phase of the analysis, so that fifty to one hundred samples could be disinterestedly titrated.

RESULTS

During the first two or three days of caffeine administration, all subjects reported stimulation with slight muscular tremors and a lessened sense of fatigue. Two subjects noted insomnia immediately upon retiring, but this was never severe enough to require sedation. Later they noted no stimulation, but did report that they slept longer and more soundly. Approximately one-half of the subjects reported diuresis during the early period of the administration. The lack of stimulation on repeated doses agrees with the findings of Eddy and Downs,[13] who noted an increase in the reaction time in habituated subjects.

In twenty-one out of thirty-eight trials, headache resulted which was as severe as the subject had ever had. In eleven trials the headache was definite, but not severe enough to necessitate treatment. In six experiments on four subjects very slight or no headache resulted. The headache-resistant group did not include any of the original abstainers from caffeine. In three of these re-

sistant subjects, the icteric index was above the normal range[23, 11, 8] and the sclerae of M. B. with an index of 23 were yellow-tinged. These three subjects have noted that they are generally free of headache of any type. However, subject M. B. with idiopathic jaundice had a typical histamine headache of 12 minutes' duration when given 0.1 mg. of histamine diphosphate intravenously. Both of the two female subjects developed severe headache which required treatment.

Characteristics of the Headache: The symptomatology of the headache is remarkably constant and may be summarized as follows: On the day of withdrawal, lethargy was usually noticeable in the morning, while a feeling of cerebral fullness occurred about noon. Actual headache usually began in the early afternoon and reached a peak three to six hours later. The subjects localized the headache as occipital or central at the onset, which then in most cases became generalized and throbbing in character. In six subjects the headache was consistently accompanied by some degree of nausea, and in two subjects vomiting occurred. In four subjects the headache was consistently accompanied by a serous rhinorrhea of such severity that they at first thought that an acute upper respiratory infection was impending. Subjects also complained of mental depression, drowsiness, yawning, and disinclination to work.

During the incipient stage of the headache the patients' ears were usually intensely hyperemic, but later if the patients became nauseated, the ears would pale and generalized pilomotor activity was observed. The headache, when well established, was intensified by bending over or by straining, as at stool (Valsalva's experiment). Relaxation, such as napping, made the headache worse, while violent physical exercise (running upstairs), with the exception of one subject, intensified the headache. Jugular vein compression with the resultant increase in cerebrospinal fluid pressure produced the usual feeling of cerebral fullness, but did not intensify the headache.

Treatment of the Headache: Headache produced by caffeine withdrawal was found to respond to treatment by caffeine or acetylsalicylic acid. When caffeine was used to relieve the headache, the subjects sometimes complained of secondary headache the next day. While benzedrine sulfate gave relief for two to six hours, in four of six subjects the headache recurred in an intensified form. Amyl nitrite afforded temporary relief in one subject, but this was then followed by a short period of exacerbation, after which the headache continued as before. In severe headache with nausea and vomiting, oxygen inhalation afforded definite relief in two subjects. If given early enough in the course of the headache, the most effective treatment was caffeine.

Blood Findings: In Table I are given the results of determinations of serum calcium, serum inorganic phosphorus, serum protein, and hematocrit during the normal and administration periods, at the time of the headache, and on the day following when the subjects were free of headache or had received caffeine in treatment of a secondary headache. Under each column are given the averages and standard deviations of all the data. Values obtained during the administration of caffeine are not tabulated separately because these values were comparable to the normal values. The serum protein and the hematocrit were found to be generally lower at the time of the headache in all but three

of the stimulant action of caffeine, and in one of these subjects this again occurred when another caffeine-withdrawal headache was attempted. It would thus appear that the true migraine headache is apt to occur during the period

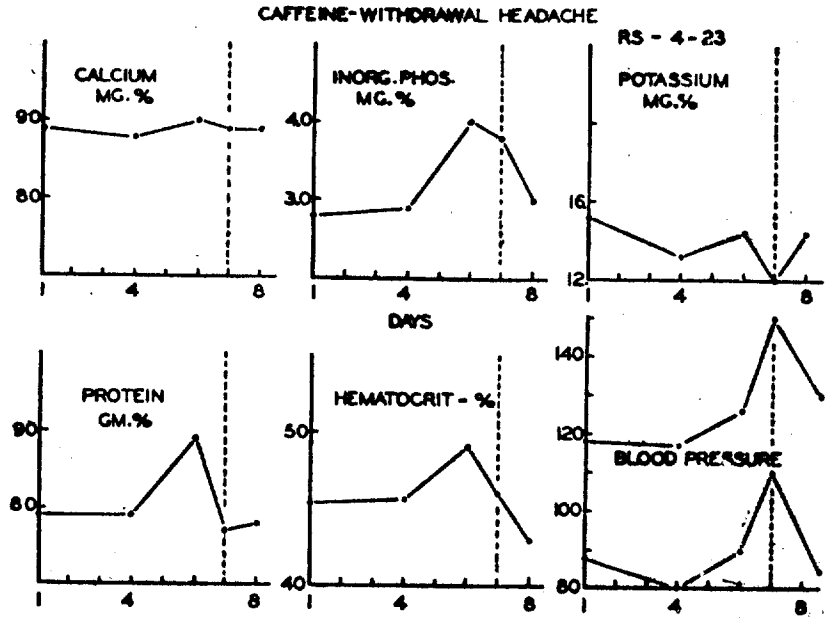

Fig. 1.

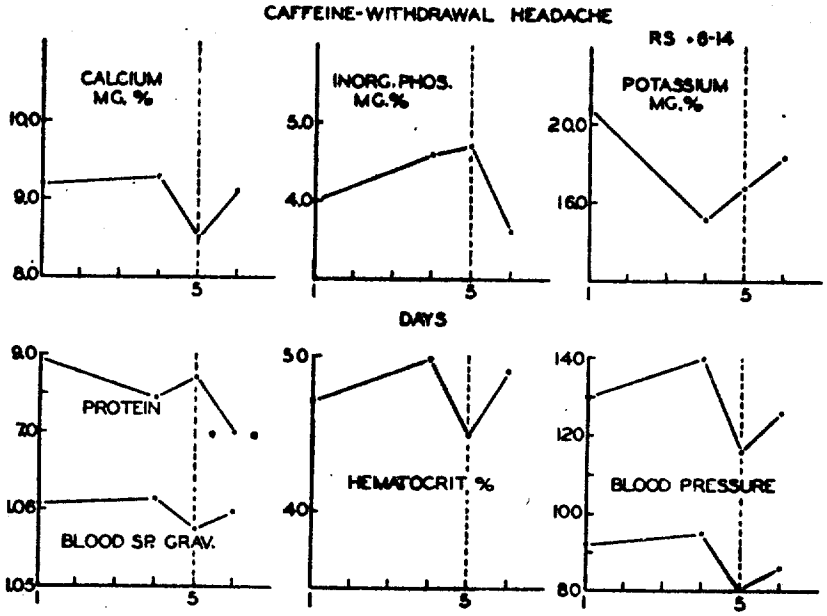

Fig. 2.

of caffeine overstimulation,* while the withdrawal of caffeine results in a distinct type of generalized headache. In a recent study[7] of migraine headache, we found no consistent changes in the serum calcium or phosphorus. A slight

*This led to an erroneous statement in the original Proceedings report.[1]

rise in hematocrit and total proteins was present at the time of the headache. The change in blood chemistry and blood volume found in this study would, hence, be another point of dissimilarity between the two types of headache.

That the caffeine-withdrawal headache is due to changes in cerebrospinal fluid pressure or volume is doubtful, since bilateral jugular compression which raises cerebrospinal fluid pressure does not intensify the headache. Amyl nitrite, however, relieves the headache during the short period of relaxation of the peripheral and splanchnic vascular tone. While the present data, due to the inadequacy of present methods, must necessarily be inconclusive, there are numerous findings that would be in accord with the hypothesis that this headache might result from an increase in effective arterial blood volume without a corresponding diminution in peripheral vascular tone, namely, the gradual onset of cerebral fullness, hyperemia of the ears, lowered serum proteins, the occurrence upon withdrawal of a diuretic drug, and the response to drugs and physical measures affecting headache.

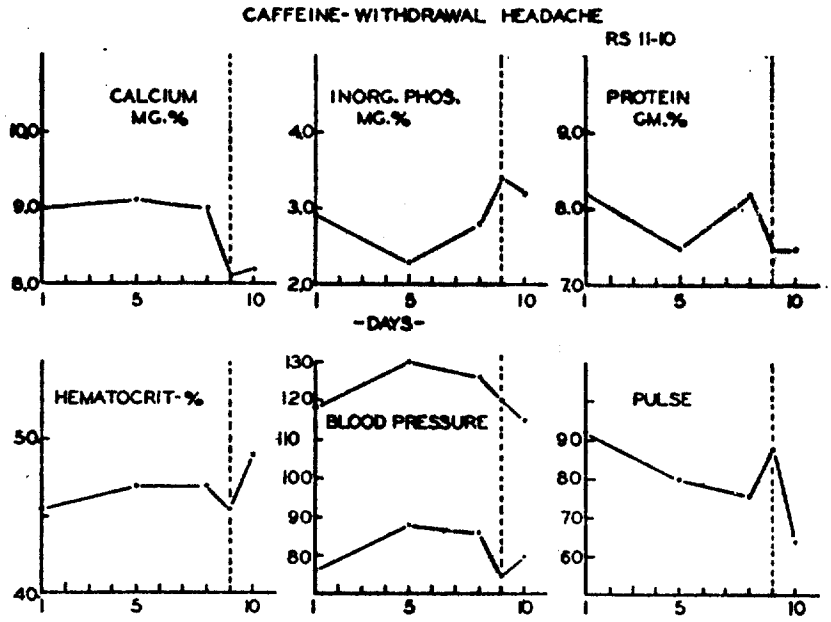

Fig. 3.

It is known from the work of Wolff[14, 15] that many headaches, including histamine and migraine headaches, are accompanied by an increased excursion of the cerebral blood vessels. This may occur reflexly or passively by changes in the volume or pressure of any one of the three semifluid media within the cranial vault, namely, the cerebrospinal fluid, the brain substance, or the blood. Since the first two media are relatively constant, and since the functional pathology of most headaches is known to reside in the vascular wall, the cause of the pain must be either a reflex dilatation of the blood vessels or an increased excursion of the vessels due to the two other factors which could affect the caliber of the cranial blood vessels, namely, the arterial blood volume or the pressure of the surrounding media. Bazett[16] has estimated the *arterial* blood volume from

anatomic measurements of the mesenteric vessels, and his observations indicate that the arteries probably contain a relatively small proportion (perhaps less than 20 per cent) of the total blood in a given region of the circulation. Thus, while the changes in blood volume demonstrated in this study are small, they may have exaggerated significance when combined with changes in cardiac output and peripheral vascular tone.

Since all factors must be considered, there remains the possibility that this headache might be due to direct or reflex dilatation of the cerebral vessels by the withdrawal of caffeine. Hirschfelder[17] has shown that caffeine tends to dilate the cerebral vessels if given intravenously. Oral caffeine is, however, believed to constrict the cerebral vessels, and this has been correlated by Sutherland and Wolff[18] with the observation that caffeine decreases the excursion of the cerebral blood vessels when used in the treatment of migraine headache.

The caffeine-withdrawal headache probably typifies one of the so-called "common headaches." In this respect it may be of value as an experimental headache. It may also provide a means of studying analgesic drugs by affording a method of producing predictable headaches. This study also provides a plausible explanation for the hitherto empirical addition of caffeine to many headache remedies.

The authors wish to express gratitude to the numerous subjects who aided in these experiments.

SUMMARY

In 55 per cent of thirty-eight trials on twenty-two subjects, headache as extreme in severity as the subjects had ever experienced was produced by the sudden withdrawal of caffeine. In 29 per cent of the trials the headache was definite, but did not require treatment. In 16 per cent of the experiments little or no headache resulted. The headache is without scotomas, slow in onset, central in origin, becoming generalized after four to six hours; and it may be accompanied by nausea and vomiting. In migraine subjects the headache differs from their typical migraine headache. The blood studies indicate that a lowered serum calcium, an elevated serum phosphorus, and possibly an increase in blood volume accompany the headache.

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Rates, Interview, and Pathology Study of Cancer of the Urinary Bladder in New Orleans, Louisiana [1]

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SUMMARY—An incidence, interview, and pathology study of cancer of the urinary bladder in whites and Negroes was carried out in New Orleans, Louisiana, from 1958–64. A total of 702 patients were identified with a presumptive or confirmed diagnosis of tumor of the bladder. The physicians in practice in New Orleans initially diagnosed the cancers of the patients in this study. Insofar as possible, pathologists at the National Cancer Institute reviewed the diagnoses. The reviewers aimed to make as certain as possible the diagnosis of the neoplastic lesion, the site of its origin, and its histologic type. In the pathology study the following patients initially reported as having bladder cancer were not included: 2.3% with clinical diagnoses only, 1.1% with the histologic diagnosis made by the New Orleans pathologists but not reviewed by National Cancer Institute pathologists, and 6.7% with a diagnosis of primary cancer in the bladder that was not confirmed after review. The histologic types of 22 (3.5%) of the 631 cancers in the pathology study were changed after review; 92% were transitional cell carcinomas (29% Grade 1; 39% Grade 2; and 24% Grade 3); 4.3% were squamous cell carcinomas; 1.1% were adenocarcinomas; 1.7% were undifferentiated cell carcinomas; 0.6% were sarcomas; and 0.3% were malignant tumors, unclassified type. Negro females (49) had larger percentages of squamous cell carcinoma, adenocarcinoma, and undifferentiated cell carcinoma than the other groups, and Negro males had a larger percentage of squamous cell carcinoma than the white males. Age-standardized incidence rates (new cases of bladder cancer per 100,000 per year) were determined for the three parishes of New Orleans (St. Bernard, Jefferson, and Orleans) combined. There were 564 patients, 97.2% with histologic diagnosis of their cancer. In the 6 years 1958–64, incidence rates ranged from 14.3–25.2 for white males, 7.5–15.9 for Negro males, 5.5–10.2 for white females, and 5.5–8.7 for Negro females, indicating considerable yearly variation. The incidence rate for bladder cancer for the total 6-year period was 11.4, *i.e.*, 19.0 for white males, 11.2 for Negro males, 7.4 for white females, and 7.2 for Negro females. The corresponding rates for 1947, as determined in the study by Dorn and Cutler, were 21.2,

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i.e., 34.6, 7.0, 17.6, and 14.6. An interviewer questioned 493 patients with bladder cancer (98.8% histologically confirmed) and 527 control patients about factors that might have influenced their disease. Cystitis, in all males, and prostatic diseases in white males were more frequent for patients with cancer of the bladder than for the controls. The ratios of "Observed" to "Expected" cases in white male patients from the incidence rates study were excessive for sailors and deckhands, tinsmiths and coppersmiths, machinists, civil engineers, plumbers, structural metal workers, barbers, and carpenters. Exposure to dusts, especially chromate dusts, was slightly more frequent in white male cancer patients than in the controls. However, doubts about the validity of some of the methods of study concerned with these factors left us unwilling to conclude that the associations observed were related to the causes of cancer of the bladder. There was a significant correlation between bladder cancer and tobacco use, all forms combined, among males only; however, this correlation did not exist when data were analyzed by type of tobacco or amounts used. Bladder cancer in our study was not associated with the history of amounts of total daily fluid ingested, alcoholic or nonalcoholic beverages, or various types of coffees, or with dietary habits, or preparations for the hair and scalp.—J Nat Cancer Inst 41: 683–709, 1968.

CANCER OF the bladder was studied from 1958–64 in New Orleans where we determined the age-adjusted incidence rates, examined and classified the pathologic material, and interviewed patients for significant environmental factors. We also compared our rates with those of Dorn and Cutler, obtained in 1947–48 for cancer of the bladder in 10 U.S. cities including New Orleans. They reported a rate two to three times larger in New Orleans than in Birmingham, Atlanta, or Dallas (*1*) and at least one-third larger than in Chicago, Detroit, Philadelphia, Pittsburgh, Denver, and San Francisco.

MATERIALS AND METHODS

During our 6-year study (Feb. 1, 1958, through Jan. 31, 1964), 702 patients were identified with a presumptive or confirmed diagnosis of tumor of the bladder (tables 1, 10).

The techniques for identifying patients with these tumors were about the same as in the study by Dorn and Cutler (*1*). The records of practicing physicians, hospitals, the Tumor Registry, and the Office of Vital Statistics were the basic sources for finding cases. In our study, the records of pathology laboratories were also consulted, but all patients found in this way had also been identified through the records of hospitals and physicians. The hospitalized patients were drawn from Charity Hospital, a large State hospital, the Veterans Administration Hospital, the U.S. Public Health Service Hospital, and 8 private hospitals with and without university affiliations. The patients were identified as whites and Negroes from information on the hospital record.

The patients for the incidence study were all residents of New Orleans proper; that is, the parishes of Orleans, St. Bernard, and Jefferson. The patients for the interview study were also from these parishes and from 20 additional parishes in Louisiana;[4] 10 patients came from the States of Alabama, Arkansas, Florida, Mississippi, and Texas. A trained social worker conducted the interview following the questionnaire shown on pages

[4] Assumption, Avoyelles, Concordia, East Baton Rouge, Lafayette, Lafourche, Livingston, Natchitoches, Plaquemines, St. Charles, St. John the Baptist, St. Landry, St. Martin, St. Mary, St. Tammany, Tangipahoa, Terrebonne, Vermilion, Washington, West Feliciana.

TABLE 1.—Review of pathologic diagnosis of cases of tumor of urinary bladder reported during 1958–64 in New Orleans

Cases	Total	Male		Female	
		White	Negro	White	Negro
Total	*702**	*365*	*87*	*190*	*60*
Clinical diagnosis only	16	5	—	9	2
Histologic slides not available for NCI review	8	2	1	2	3
With NCI review of histologic material	*678*	*358*	*86*	*179*	*55*
Diagnosis of cancer not confirmed	24	14	2	7	1
Cancer considered to be not primary in bladder	23	7	6	5	5
Cancers with histologic material available considered to be primary in bladder after NCI review	*631*	*337*	*78*	*167*	*49*

**See* table 10, p. 690, for 91 patients not included in study period and in geographic area.

693–697. Two of the authors, A.S.R. and H.L.S., made yearly trips to New Orleans where they reviewed the histopathologic material and medical records and classified the tumors. The populations used in the calculation of the rates were from U.S. Census Bureau statistics for New Orleans for 1950 and 1960 (*2, 3*).

RESULTS

Pathology

The pathologists from the National Cancer Institute (NCI) examined the medical records of the 702 cases reported to be tumors of the bladder and reviewed the histopathologic material of 678, or 96.6% (table 1).

Twenty-four cases of cancer of the bladder were accepted for the rates and interview study, even though histologic slides were not available for review by NCI pathologists. Sixteen of these had been diagnosed clinically only and were accepted largely on the basis of the report of the cystoscopic examination; the remaining 8, with biopsies, were accepted on the basis that the histologic material from them had been diagnosed cancer of the bladder by the New Orleans pathologists.

We used a classification of cancer of the bladder modified from that reported by Mostofi (*4*):

Papilloma: Papillary not invasive, with epithelium similar in all respects to that of the normal bladder. We encountered no example of this lesion in our study.

Transitional cell carcinoma:

Grade 1.—Papillary, not infiltrative,[5] composed of delicate fronds with slight histologic abnormalities of epithelium, such as increased thickness, nuclear pleomorphism, and hyperchromatism, and occasional mitoses.

Grade 2.—Papillary, or infiltrative,[5] or both, composed of delicate and coarse fronds or stubby papillary processes or inverted papillary formations, with increased numbers of mitotic figures and accentuation of the histologic abnormalities of epithelium noted under Grade 1.

Grade 3.—Papillary, or infiltrative,[5] or both, with pronounced accentuation of the abnormal features noted under Grade 1 and Grade 2.

Squamous cell carcinoma
Adenocarcinoma
Undifferentiated cell carcinoma
Sarcoma
Malignant tumor, type unclassified

After review of the histologic slides of 678 patients, 47, or 6.9%, were not included: In 24 the diagnosis of cancer could not be confirmed; in 23 with cancer there was doubt about the primary site and these were not included. Table 2 gives the histologic classification of the remaining 631 cancers. This classification agreed with the initial classification by the New Orleans pathologists in most instances. Changes were made in 22 of the 631 cases, or 3.5% (table 3). Six lesions classified as papilloma were, in our opinion, Grade 1 transitional cell carcinoma. Also, several tumors were changed from transitional cell carcinoma to squamous cell carcinoma and from squamous cell carcinoma to transitional cell carcinoma. These changes reflect differences in classification and nomenclature and do not alter the diagnoses of primary bladder neoplasm.

[5] Our criterion for infiltration was the presence of cancer within the musculature of the bladder.

TABLE 2.—Histologic classification of bladder cancers after review by NCI pathologists

Group	Total		Transitional cell carcinoma		Squamous cell carcinoma		Adenocarcinoma		Undifferentiated cell carcinoma		Sarcoma		Malignant tumor, type unclassified	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Total....	*631*	100.00	580	92.0*	27	4.3	7	1.1	11	1.7	4	0.6	2	0.3
White male....	*337*	100.00	323	96.0	4	1.1	3	0.9	4	1.1	2	0.6	1	0.3
Negro male....	*78*	100.00	69	88.5	6	7.6	1	1.3	1	1.3	1	1.3	—	—
White female...	*167*	100.00	155	92.9	8	4.7	—	—	3	1.8	—	—	1	0.6
Negro female...	*49*	100.00	33	67.5	9	18.3	3	6.1	3	6.1	1	2.0	—	—

*Grade 1, 29.4%; Grade 2, 38.7%; and Grade 3, 23.9%; totaling 92.0%.

Most of the 631 cancers, 580, or 92%, were transitional cell carcinomas. The Negro patients, especially the females, had a lower proportion of transitional cell carcinoma than the white patients, but a higher proportion of squamous cell carcinoma. The excess of squamous cell carcinoma for both male and female Negroes was statistically significant. Negro females also had higher proportions of adenocarcinoma and undifferentiated cell carcinoma than the other 3 groups of patients. The results of grading the transitional cell carcinomas by the NCI pathologists are shown in table 4. Changes made in grading after review are not shown, since the reviewing pathologists used grading criteria that were different from some of the New Orleans pathologists'. In the Negro as compared with the white, proportionately more of the transitional cell carcinomas were in Grade 3.

In 38% of cases of transitional cell carcinoma the biopsy specimen consisted only of papillary fronds or a superficial portion of the tumor without underlying connective tissue and muscle, and was therefore not adequate to evaluate the presence or absence of infiltration. The comparatively large number of specimens with tissue inadequate for the determination of infiltration indicates the difficulties encountered in the evaluation of this feature in biopsy material obtained in routine urologic practice. In the 62% with biopsies adequate for this determination, approximately one-half showed infiltration (table 5).

Incidence Rates

The incidence study included 564 residents of Orleans, St. Bernard, and Jefferson Parishes, New Orleans, whose bladder cancers were first diagnosed during 1958-64 (tables 6 and 7). From the 702 patients reported as having tumor of the bladder, 91 were excluded either because they resided outside the study area or because the diagnosis of cancer of the bladder was made before the start of the study, or for both reasons. Forty-seven patients not considered to have primary cancer of the bladder after histologic review were also excluded (table 6).

Of the 564 patients used for calculating incidence rates, cancer was diagnosed clinically only in 16, and histologic slides from 8 were examined by New Orleans but not by NCI pathologists. Alto-

TABLE 3.—Changes in histologic classification made by NCI pathologists

Initial classification	Total	Final classification: Transitional cell carcinoma	Undifferentiated cell carcinoma	Squamous cell carcinoma	Adenocarcinoma	Malignant tumor, type unclassified
Total.....	*22*	*10*	*6*	*2*	*2*	*2*
Papilloma.....	6	6	—	—	—	—
Transitional cell carcinoma...	9	—	5	2	2	—
Undifferentiated cell carcinoma.....	4	2	—	—	—	2
Squamous cell carcinoma.....	3	2	1	—	—	—

TABLE 4.—Grade and the infiltration of the transitional cell carcinomas of the bladder

	Total	Infiltration	No evidence of infiltration	Tissue inadequate to evaluate infiltration
Total 580				
White male	*323*	*87*	*119*	*117*
Grade 1	103	—	53	50
Grade 2	152	29	60	63
Grade 3	68	58	6	4
Negro male	*69*	*29*	*14*	*26*
Grade 1	18	—	5	13
Grade 2	23	7	8	8
Grade 3	28	22	1	5
White female	*155*	*42*	*42*	*71*
Grade 1	55	—	17	38
Grade 2	61	11	24	26
Grade 3	39	31	1	7
Negro female	*33*	*14*	*13*	*6*
Grade 1	9	—	9	—
Grade 2	8	1	2	5
Grade 3	16	13	2	1
Recapitulation				
Total	*580*	*172*	*188*	*220*
Grade 1	185	—	84	101
Grade 2	244	48	94	102
Grade 3	151	124	10	17

TABLE 5.—Analysis of infiltration in review of 580 transitional cell carcinomas of the bladder

Group	Total		Infiltration		No evidence of infiltration		Tissue inadequate to evaluate infiltration	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Total	*580*	100.0	*172*	29.6	*188*	32.4	*220*	38.0
White male	323	100.0	87	26.9	119	36.8	117	36.3
Negro male	69	100.0	29	42.0	14	20.3	26	37.7
White female	155	100.0	42	27.1	42	27.1	71	45.8
Negro female	33	100.0	14	42.4	13	39.4	6	18.2

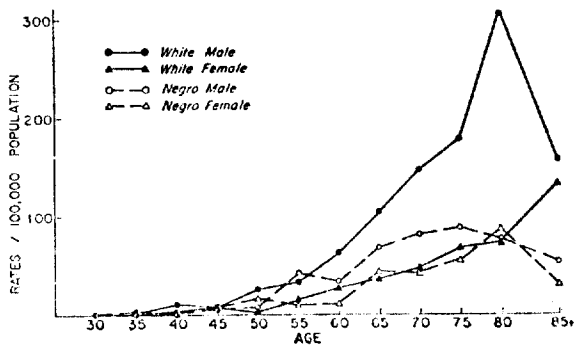
TABLE 6.—Derivation of cases for incidence study, New Orleans, 1958–64

Bladder cancer patients	Total	Male		Female	
		White	Negro	White	Negro
Total reported	702	365	87	190	60
Out of study period, or area or both	91*	54	14	21	2
Cancer unconfirmed after review of diagnosis	47	21	8	12	6
Balance in incidence study	564	290	65	157	52

*These cases are described in table 10 on p. 690.

ether, the lesions of 97.2% of the patients were diagnosed histologically. The white and nonwhite populations of New Orleans were estimated for February 1, 1961, the midpoint of the study, from the published U.S. census data (*2*, *3*).

Tables 7 and 8 and text-figure 1 show the incidence rates of bladder cancer in New Orleans. The rate is about the same for white and Negro females, and is more than 1½ times larger for white than for Negro males. The male-to-female



TEXT-FIGURE 1.—Incidence rates of cancer of the urinary bladder by age, ages 30 and over, New Orleans, 1958–64.

ratio is more than 2 for white patients and 1½ for Negroes.

Table 8 compares rates in New Orleans in 1947 and 1958–64 with each other and with the 1947–48 rates for the 10 U.S. cities combined.

The 1958–64 rates were from data for the New Orleans Standard Metropolitan Statistical Area (Orleans, St. Bernard, and Jefferson Parishes), while those for 1947 were from data for Orleans Parish alone. We ascertained that 64% of males in Orleans Parish and 72% in all 3 parishes combined were white males. To estimate the extent of difference in rates due to the nonidentical areas used for the comparison, we calculated the average annual age-adjusted rate for white males in Orleans Parish. It was 18.5 compared to 19.0 for the same group in the New Orleans Standard Metropolitan Statistical Area. Thus from a practical viewpoint the two sets of rates are comparable, at least for the large group of white males, even though the rates are not based on identical populations.

Table 8 shows that the New Orleans rates for 1958–64 were considerably lower than for 1947 and generally similar to those for the entire 10-cities study in 1947–48. They were about 50% higher than rates in Atlanta or Birmingham (*1*). It might tentatively be assumed that the 91 New Orleans cases out of the study period or area and the 47 cases excluded with cancer unconfirmed after review of the diagnosis (table 6) were in categories of cases that might have been retained in the 1947 study. Even if this assumption is correct, rates of bladder cancer in New Orleans in the 1958–64 study still decreased at least 30% as compared with the 1947 study.

We explored factors that might explain the apparent decrease of rates in New Orleans between

TABLE 7.—Number of new cases of cancer of the urinary bladder: incidence rates per 100,000 population, per year, by age, sex, and race, 1958–64

Age	Total		Male				Female			
			White		Negro		White		Negro	
	Number	Rate	Number	Rate	Number	Rate	Number	Rate	Number	Rate
0–4	—	—	—	—	—	—	—	—	—	—
5–9	—	—	—	—	—	—	—	—	—	—
10–14	1	0.2	—	—	—	—	1	0.6	—	—
15–19	1	0.3	1	0.8	—	—	—	—	—	—
20–24	2	0.6	—	—	1	2.3	—	—	1	1.8
25–29	4	1.2	—	—	1	2.3	1	0.9	2	3.7
30–34	1	0.3	—	—	—	—	1	0.7	—	—
35–39	7	1.8	3	2.1	2	4.1	1	0.7	2	1.8
40–44	21	6.4	12	10.2	1	2.4	6	4.9	2	4.3
45–49	27	8.6	9	8.0	3	7.6	11	9.4	4	8.8
50–54	40	13.7	26	25.8	3	8.0	4	3.6	7	16.3
55–59	63	24.5	30	33.7	14	41.0	15	15.4	4	10.8
60–64	75	38.0	43	61.7	8	34.7	21	26.7	3	11.6
65–69	98	62.1	51	102.7	13	66.9	24	36.4	10	43.9
70–74	88	79.7	48	146.3	10	80.9	23	47.2	7	42.6
75–79	67	97.3	33	177.1	6	87.6	22	67.7	6	55.2
80–84	46	136.8	27	304.5	2	76.5	13	74.3	4	86.0
85+	23	113.7	7	155.1	1	54.3	14	132.4	1	30.3
All ages:										
Crude	564	10.6	290	16.4	65	8.3	157	8.3	52	6.9
Adjusted*		11.4		19.0		11.2		7.4		7.2

*Age-adjusted to total population of continental United States, 1950 (*2*).

TABLE 8.—Bladder cancer rates (cases per 100,000, per year)* for the population of New Orleans, 1947 and 1958–64 and for 10 large U.S. cities surveyed in 1947–48

Study	Total rate	Male		Female	
		White	Nonwhite	White	Nonwhite
New Orleans, 1958–64	11.4	19.0	11.2†	7.4	7.2†
New Orleans, 1947‡	21.2	34.6	7.0	17.6	14.6
10 cities, 1947–48‡	12.5	18.6	7.0	8.0	7.9

*All rates were age-adjusted to the USA 1950 Standard Population (8). † Negro only; about 90% of U.S. nonwhites are Negroes. ‡(1).

the 2 surveys. Age-adjusted rates for each of the 6 years of the 1958–64 study are shown in table 9.

Since data collection methods were constant from 1958–64, the considerable yearly fluctuations may have been due to random variation or possibly some environmental factor. The yearly fluctuation for white males shown in table 9 is no greater than may occur between three and four in 100 times by chance alone. Aspects of collection of the data that might clarify reasons for the 1962–63 difference were scrutinized. Some variation in numbers of cases from month to month is usual, and if few diagnoses were made at the end of 1 year and many at the beginning of the next year, this might lead to adjacent low-rate and high-rate years. We did not find such an effect. The numbers of cases of bladder cancer in each hospital in each year were examined also. Hotel Dieu Hospital and Veterans Hospital, 2 of the 11 participating hospitals, reported 12 and 10 bladder cancers, respectively, in 1963. However, in the other 5 years examined, Hotel Dieu Hospital reported only 23 and Veterans Hospital only 17 bladder cancers, with the numbers of cases about equally distributed, averaging fewer than half as many per year as in 1963. All patients were New Orleans residents and we do not know the explanation for the excess of white males with bladder cancer in the two hospitals in 1963. Furthermore, we cannot explain the reason for the apparent decrease of rates in New Orleans from 1947 to 1958–64.

Interview Study

All interviews were conducted in New Orleans hospitals by an experienced social worker (A.S.F.). Patients were located through pathology and hospital records, and by consulting at frequent intervals the cooperating physicians in private practice, members of their office staffs, and pathology laboratory and hospital staff members. Text-figure 2 shows the questionnaire. There were 493 patients with cancer in the interview study including 402 from the incidence study and 91 others (*see* footnote, table 10). We did not interview 162, or 28.7%, of the incidence rates cases. Failure to interview (ranging from 13.5% in Negro females to 42% in white females) was due to lack of notification about a patient until after hospital discharge or death, unwillingness of a patient or physician to allow an interview, or a patient's severe physical or emotional illness. Such failures are always present, but not always recorded in published reports. We do not know what are the effects on the results of our study or other interview studies of the bias produced by the incomplete data.

TABLE 9.—Age-adjusted cases of bladder cancer in New Orleans 1958–64, by year of study, compared with 1947

Group	1958–63							1947	
	Number of cases	1958	1959	1960	1961	1962	1963	Number of cases*	Rate
Total	*564*							*105*	
White male	290	20.2	16.9	17.6	18.6	14.3	25.2	53	34.6
Negro male	65	10.4	13.2	7.5	15.9	10.3	10.0	4	7.0
White female	157	10.2	8.0	6.1	6.5	8.4	5.5	37	17.6
Negro female	52	6.4	7.7	8.7	7.1	8.0	5.5	11	14.6

*(6).

TABLE 10.—Derivation of patients with bladder cancer included in the interview study

Patients	Total	Male		Female	
		White	Negro	White	Negro
Total with bladder cancer	702	365	87	190	60
Subtract patients not interviewed	162	79	10	66	7
Subtract discards after review of diagnosis	47	21	8	12	6
Balance in interview study	493*	265	69	112	47

Patients	Total	Male		Female	
		White	Negro	White	Negro
*The following 91 patients were included in the interview study but not in the rates study:	91	54	14	21	2
1) Cancer diagnosed before 2/1/58	25	15	1	9	–
2) Not residing in New Orleans	56	32	12	10	2
3) In both categories	10	7	1	2	–

Six patients had clinical diagnoses only and three had pathologic diagnoses made in New Orleans but not reviewed by NCI pathologists. Altogether there was histologic confirmation of the diagnosis of bladder cancer in 487, or 98.8%, of the cancer patients who were interviewed.

The study included 527 control patients without cancer of the bladder, interviewed in the same hospitals (table 11) and during the same period of study. There was equal representation in cancer and control groups of ward, semiprivate, and private patients, and also of patients who were residents of metropolitan New Orleans. Over 90% of controls had diagnoses unrelated to the genitourinary tract or to neoplastic disease (table 12). Some of the patients first selected for interview following the interview of a cancer patient could not be included in the study because the patient or doctor withheld permission or because the patient suffered from a severe physical or emotional illness. The next immediately available control was selected for interview. Finally, the control group was larger by 34 patients than the bladder cancer group, in part because some cancer patients were excluded from the study after review of the histologic diagnosis. Proportions of controls and cancer patients were similarly distributed in age, sex, and race categories (table 13).

The data obtained at interviews are the basis for the comparisons between cancer and control patients.

PAST MEDICAL HISTORY

Information obtained about specific illnesses and operations is presented in table 14. The correlation between cancer of the bladder and the history of calculi was not at a consistently significant level. The correlation with the history of cystitis was significant ($P < 0.05$) for white and Negro males. Among white males there was a strong correlation between cancer of the bladder and the history of conditions that presumably produced obstruction to the urinary flow ($P < 0.005$). This positive correlation was due almost entirely to various types of prostatic disease, such as prostatitis, prostatic hyperplasia or hypertrophy, indication for prostatectomy, prostatism, and "prostate trouble," especially during the 3 years just before the diagnosis of cancer. The other factors that caused obstruction to the urinary flow diverticulitis of the bladder and urethral stricture, were not correlated with cancer of the bladder. There were more patients with diabetes among the controls than in the groups with cancer of the bladder except for Negro females. This negative correlation with the history of diabetes can probably be dismissed from consideration, since the method of selecting patients to interview as controls is likely to have favored the inclusion of hospitalized diabetic patients in the control groups. The possibility of other artifacts has been considered, and some of these are discussed elsewhere in the paper.

TABLE 11.—Number of interviewed patients with cancer of the bladder and control patients (all patients classified by sex and race) in hospitals in New Orleans, 1958–64

	Total	Baptist	Charity	Flint-Goodridge	Hotel Dieu	Mercy	Ochsner	Sara Mayo	Touro	USPHS	Veterans	West Jefferson
Total:												
Cancer	493	54	170	15	50	51	39	3	64	8	36	3
Control	527	45	195	16	40	51	36	1	95	7	39	2
White male:												
Cancer	265	36	56	—	35	35	26	—	40	8	27	2
Control	272	30	62	—	29	33	23	—	57	7	29	2
Negro male:												
Cancer	69	—	49	11	—	—	—	—	—	—	9	—
Control	77	—	55	11	—	1	—	—	—	—	10	—
White female:												
Cancer	112	18	25	—	15	16	13	—	24	—	—	1
Control	123	15	30	—	11	17	13	—	37	—	—	—
Negro female:												
Cancer	47	—	40	4	—	—	—	3	—	—	—	—
Control	55	—	48	5	—	—	—	1	1	—	—	—

TABLE 12.—Control patients: genitourinary diseases, tumors, and other diseases

Group	Total	Calculi in kidney, ureter, or bladder	Urinary bladder			All other genito-urinary diseases	Other tumors*		All other diseases †
			Cystitis	Diverticulitis, trauma, mechanical interference with flow	Leukoplakia		Benign	Malignant	
Total	*527*	*4*	*2*	*13*	*2*	*2*	*8*	*11*	*485*
Male, white	273	4	1	6	1	1	3	3	254
Male, Negro	77	—	1	1	—	1	—	2	72
Female, white	122	—	—	6	1	—	1	4	110
Female, Negro	55	—	—	—	—	—	4	2	49

*None of the tumors was primary in the urinary bladder.

†Examples are:

Acrodermatitis
Arteriosclerotic heart disease
Asthmatic bronchitis
Auricular fibrillation
Bronchiectasis
Cirrhosis of liver
Coronary thrombosis
Diabetes
Emphysema
Essential hypertension
Fracture of femur
Fracture of hip
Gallbladder disease
Gastric or duodenal ulcer
Gout
Inguinal hernia
Meckel's diverticulum
Megacolon
Myocardial disease
Osteomyelitis
Pancreatitis
Parkinsonism
Peripheral vascular disease
Psoriasis
Pulmonary edema
Traumatic injury of biceps

TABLE 13.—Age distribution of New Orleans patients, interview study of patients with cancer of urinary bladder and control patients, 1958–64

Groups	Total		Frequency distribution by ages at diagnosis																			
			Less than 35		35–39		40–44		45–49		50–54		55–59		60–64		65–69		70–74		75+	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Total cancer	*493*	100.0	*6*	1.2	*5*	1.0	*19*	3.9	*29*	5.9	*37*	7.5	*51*	10.3	*73*	14.8	*91*	18.5	*73*	14.8	*109*	22.1
Male, white	265	100.0	1	0.4	2	0.8	13	4.9	16	6.0	21	7.9	23	8.7	43	16.2	47	17.7	40	15.1	59	22.3
Male, Negro	69	100.0	1	1.4	1	1.4	—	—	2	2.9	4	5.8	15	21.7	10	14.6	15	21.7	10	14.6	11	15.9
Female, white	112	100.0	2	1.8	1	0.8	4	3.6	7	6.2	4	3.6	9	8.0	17	15.2	20	17.9	17	15.2	31	27.7
Female, Negro	47	100.0	2	4.3	1	2.1	2	4.3	4	8.5	8	17.0	4	8.5	3	6.4	9	19.1	6	12.8	8	17.0
Total control	*527*	100.0	*8*	1.5	*6*	1.0	*22*	4.2	*33*	6.3	*42*	8.0	*58*	11.0	*78*	14.8	*98*	18.6	*71*	13.5	*111*	21.1
Male, white	273	100.0	1	0.4	3	1.0	13	4.8	17	6.2	21	7.7	25	9.2	47	17.2	51	18.7	36	13.2	59	21.6
Male, Negro	77	100.0	1	1.3	1	1.3	1	1.3	2	2.5	9	11.7	16	20.8	11	14.3	15	19.5	12	15.6	9	11.7
Female, white	122	100.0	3	2.5	1	0.8	6	4.9	7	5.7	4	3.3	10	8.2	16	13.1	22	18.0	18	14.8	35	28.7
Female, Negro	55	100.0	3	5.5	1	1.8	2	3.6	7	12.7	8	14.5	7	12.7	4	7.3	10	18.3	5	9.1	8	14.5

TEXT-FIGURE 2.—Questionnaire.

1. Patient's name: Middle First | 2. M ☐ F ☐ | 3. Study No.:

4. Hospital | 5. Hospital No.: Path. No.: | 6. Date admitted: Interviewed:

7. Status: Ward ☐ Semiprivate ☐ Private ☐ OPD ☐ | 8. Blood Group:

9. Date of birth: Age: | 10. Place of birth: Patient: Mother: Father:

11. S ☐ M ☐ W ☐ D or S ☐ W ☐ N ☐ | 12. Education (Highest grade completed):

13. Residence (Start with present address and work back):
A. Name of City, Town, Village:
B. County, Parish, etc.:
C. State:
D. Residence: Inside limits of City ☐ Town ☐ Village ☐
Built-up area around City or Town: Yes ☐ No ☐ Open country: Yes ☐ No ☐
E. When did you move here? _____ _____ (This means City, Town, Village, not necessarily street)
Month Year

14., 15., and 16. Residences before this; questions were asked about a total of 4 residences.

17. Date Dx. MN: | 18. Earlier Dx. MN., if any: (Give hospital, date, type, path. no.):

19. Diagnosis: Histologic type:

Control Diagnosis:

20. Micro. conf.:
Yes ☐
Clin. only: ☐
Yes & reviewed: ☐
Source:

21. Past medical and surgical history. Questions were asked about the following Illnesses or Diagnoses, the Dates of Illness and the Doctor or Hospital: General, including tumors and surgery; Diabetes; Venereal diseases; Genitourinary disease (Cystitis, including recurrent; Diverticulitis; Other inflammatory disease; Renal colic; Kidney stones; Bladder stones; Leukoplakia; Tumors; Family history of stones).

22. Past urination history: Pus in urine; Blood in urine; Urgency; Incomplete emptying of bladder; Day and night frequencies; (for Male) Size of stream, Force of stream, Hesitancy, and Interruption of urination.

23. For married women only: Current occupation of husband _____

24. Occupational history:

OCCUPATION (Title and description of duties. Indicate if proprietor "owner". Begin with present or last job.) COMPANY NAME AND ADDRESS (Specify whether State, federal or local government)	INDUSTRY (State kind of business, product made, and whether mfg., wholesale, retail, etc.) OUTDOOR JOB (Four hours or more daily, Yes or No)	YEARS WORKED The questions were asked about all past occupations.

25. Exposure: Has there been regular continuous exposure to any of the following:

SUBSTANCE	YES	NO	SUBSTANCE	YES	NO	SUBSTANCE	YES	NO
Tar			Beta naphthylamine			Other dusts		
Exhaust fumes Cleaning agents			Arsenic Asbestos dust			Radiation		
Insecticides			Chromate dust					
Dyes			Silica dust					

In any of your jobs have you handled, been exposed to, or come in contact with any substance which you believe may be related to your present illness? Yes ☐ No ☐ If Yes, specify below. (Include medicines or home remedies if there is a history of more than occasional use).

SUBSTANCE	NATURE OF CONTACT OR EXPOSURE	EMPLOYER	DATES	
			FROM	TO

As much information as possible was elicited.

26. *Use of alcoholic beverages:*
Unit consumed, Frequency of use, Age started and age stopped: Questions were asked separately for Wine, Beer, Whisky, Rum, Gin, Brandy and Other (specified). Have you made any major changes in drinking alcoholic beverages during the past 20 years? Yes ☐ No ☐. If yes, describe the changes; state units, frequency and starting and stopping ages of major changes in use for each type of beverage: _____

27. *Non-alcoholic beverages consumed regularly:*
Type, Unit per day, Age started and Age stopped: Questions were asked separately for Coffee, Soft Drinks, Other, and Water. Have you made any major changes in consumption of non-alcoholic beverages during the past 20 years? Yes ☐ No ☐. If yes, describe the changes; state units per day and starting and stopping ages of major changes in use for each type of beverage. _____

28. Principal foods: Times per week (7 or more, 3 to 6, 1 to 2, Rarely and Never): asked about Milk, cheese, and ice cream; Meat, lean; Meat, fat; Poultry; Fish; Eggs; Citrus fruits, and leafy and yellow vegetables; Peas and beans; Potatoes, sweet; Potatoes, white; Breads, cereal foods, rice, macaroni, noodles, and spaghetti; Fats, animal; Butter; Oleo, vegetable fats and oils; Pies, cakes, candies, and puddings; Highly spiced and seasoned foods; Others (specified).

29. Do you like your foods highly seasoned? Yes ☐ No ☐ Have you made any recent major changes in food habits (types, frequency) during last 20 years? Yes ☐ No ☐ If yes, describe the changes: _____

30. *Tobacco use:*

Do you now regularly	(smoke)	(chew)	*Inhale*		*Filtered*	
	Yes	No	Yes	No	Yes	No
Cigarettes						
Cigars						
Pipe						
Chewing						
Snuff						

Have you ever regularly	(smoked)	(chewed)	*Inhale*		*Filtered*	
	Yes	No	Yes	No	Yes	No
Cigarettes						
Cigars						
Pipe						
Chewing						
Snuff						

Do you (smoke) (chew) more than one brand? Yes ☐ No ☐
What is your favorite brand?
How long have you (smoked) (chewed) this brand _____ years?

31. *Present users; cigarettes:*
How old were you when you started to smoke cigarettes regularly? _____ years.

At the present time, how many cigarettes do you smoke on the average?	Check one	How many years have you smoked at this rate?
0. Smoke cigarettes once in a while but not every day		
1. Regularly smoke cigarettes but less than 10 a day		
2. Regularly smoke from 10 to 19 cigarettes a day		
3. Regularly smoke more than 19 but less than 40 cigarettes a day		
4. Regularly smoke 40 or more cigarettes a day		

Persons who have stopped; cigarettes:
How old were you when you started to smoke cigarettes regularly? _____years.
How old were you when you finally stopped smoking cigarettes? _____years.
How many cigarettes per day were you smoking just before you decided to stop?

Average number of cigarettes per day:	Check one	Number of years
5. Smoked cigarettes once in a while but not every day		
6. Regularly smoked less than 10 cigarettes a day		
7. Regularly smoked from 10 to 19 cigarettes a day		
8. Regularly smoked more than 19 but less than 40 cigarettes a day		
9. Regularly smoked 40 or more cigarettes a day		

Why did you stop?

32. *Present users, cigars:*
How old were you when you started to smoke cigars regularly? _____years.

At the present time, how many cigars do you smoke on the average?	Check one	Number of years
0. Smoke cigars once in a while but not every day		
1. Regularly smoke 1 or 2 cigars a day		
2. Regularly smoke 3 or 4 cigars a day		
3. Regularly smoke 5 to 8 cigars a day		
4. Regularly smoke 9 or more cigars a day		

Persons who have stopped, cigars:
How old were you when you started to smoke cigars regularly? _____years.
How old were you when you finally stopped smoking cigars? _____years.
How many cigars per day were you smoking just before you decided to stop?

Average number of cigars per day:	Check one	Number of years
5. Smoked cigars once in a while but not every day		
6. Regularly smoked 1 or 2 cigars a day		
7. Regularly smoked 3 or 4 cigars a day		
8. Regularly smoked 5 to 8 cigars a day		
9. Regularly smoked 9 or more cigars a day		

Why did you stop?

33. *Present users, pipe:*
How old were you when you started to smoke a pipe regularly? _____years.
At the present time, how many pipefulls do you smoke on the average?

	Check one	How many years have you smoked at this rate?
0. Smoke a pipe once in a while but not every day		
1. Regularly smoke less than 5 pipefulls a day		
2. Regularly smoke from 5 to 9 pipefulls a day		
3. Regularly smoke from 10 to 19 pipefulls a day		
4. Regularly smoke 20 or more pipefulls a day		

Persons who have stopped, pipe:
How old were you when you started to smoke a pipe regularly? _____years.
How old were you when you finally stopped smoking a pipe? _____years.
How many pipefulls per day were you smoking just before you decided to stop?

Average number of pipefulls per day:	Check one	Number of years
5. Smoked a pipe once in a while but not every day		
6. Regularly smoked less than 5 pipefulls a day		
7. Regularly smoked from 5 to 9 pipefulls a day		
8. Regularly smoked from 10 to 19 pipefulls a day		
9. Regularly smoked 20 or more pipefulls a day		

Why did you stop?

34. *Present users:* Chewing ☐ Snuff ☐
How old were you when you started to chew tobacco or use snuff regularly? _____years.
At the present time how much tobacco do you chew or how much snuff do you use on the average?

	Check one	How many years have you chewed tobacco or used snuff?
0. Chew tobacco or use snuff once in a while but not every day		
1. Regularly chew tobacco or use snuff practically daily		

Persons who have stopped: Chewing ☐ Snuff ☐
How old were you when you started to chew tobacco or use snuff regularly? _____ years.
How old were you when you finally stopped chewing tobacco or using snuff? _____ years.
How much tobacco did you chew or how much snuff did you use per day just before you decided to stop?
Average amount of tobacco or snuff used per day:

	Check one	Number of years
2. Chewed tobacco or used snuff once in a while but not every day		
3. Regularly chewed tobacco or used snuff practically daily		

Do you swallow? Yes ☐ No ☐ Spit? Yes ☐ No ☐
Why did you stop? _____

35. *Ethnic group:* _____

36. *Interviewer's comments:* _____

Among the patients with a present diagnosis of bladder cancer and a past history of malignant tumor elsewhere, the site of the prior malignant tumor did not appear to be related to the present diagnosis of bladder cancer. A listing of the cancers follows:

	Intestines and rectum	Lung and larynx	Prostate gland	Kidney	Skin	Melanoma
Male patients:						
Cancer: White	2	2	2	1	16	1
Negro	—	—	—	—	—	—
Control: White	2	—	2	—	15	—
Negro	—	—	1	—	—	—

	Intestines and rectum	Breast	Uterus, not otherwise specified	Other genital	Skin
Female patients:					
Cancer: White	2	2	3	1	2
Negro	—	—	2	—	—
Control: White	—	2	1	1	—
Negro	—	—	—	—	—

Occupation and Industry

Occupations and industries in the life history of patients with cancer of the bladder were selected for study to identify environmental exposures not previously recognized that might be related to development of this cancer. The Alphabetical Index of Occupations and Industries, 1950 U.S. Bureau of the Census (*6*) was used to classify and code these data. The number of occupations per person was 2.1 for each group of males, 0.9 for white females, and 1.2 for Negro females, with closely similar numbers in the corresponding control groups. Percentage distributions, both of individual and grouped occupations and industries, were analyzed for cancer and control patients. The small numbers of patients in occupations and industries in the ungrouped categories, and the

TABLE 14.—Medical history of interviewed patients

Groups	Total patients	Percent* patients with none of diseases listed in headings	Percentages*								
			Genitourinary calculi	Cystitis	Obstruction to urinary flow	Leukoplakia of bladder	Gonorrhea	Other genitourinary disease	Prior benign tumor (not in bladder)	Prior malignant tumor (not in bladder)	Diabetes
Cancer:											
White male	265	39.6	12.1	12.8	18.9	0.4	10.2	3.0	6.0	9.1	4.2
Negro male	69	44.9	5.8	18.8	17.4	—	26.1	4.3	5.8	—	4.3
White female	112	32.1	3.6	32.1	2.7	1.8	0.9	7.1	18.7	8.9	8.0
Negro female	47	31.9	8.5	27.7	4.3	—	12.8	12.8	29.8	4.3	17.0
Control:											
White male	273	56.0	5.9	6.2	9.5	—	8.1	2.9	7.0	7.0	10.3
Negro male	77	48.1	2.6	5.2	10.4	—	28.6	3.9	5.2	1.3	18.2
White female	122	49.2	5.7	23.8	9.8	—	0.8	5.7	23.8	3.3	12.3
Negro female	55	62.6	—	12.7	1.8	—	9.1	5.5	23.6	—	10.9

*Percentages add to more than 100, because some patients had more than one condition.

loss of specific information in the grouped categories are discussed on page 707. We did not find clear correlations of bladder cancer with an occupation or industry.

This part of the study was extended, for white male cancer-incidence patients, by calculating the "Observed" and "Expected" numbers in several occupations (table 15). Similar figures were calculated for industries as well, but since numerous occupations were included in each industrial group, the results did not appear to be significant and are not presented.

The following occupation categories are shown in the table because of an excess of "Observed" patients with bladder cancer, *i.e.*, sailors and deckhands, tinsmiths and coppersmiths, machinists, civil engineers, plumbers, structural metal workers, barbers, and carpenters. There was also an excess among an ill-defined group, "foremen." In all instances the numbers of "Expected" patients were small, a factor that reduces confidence in the validity of the observations.

In addition to the 4 white male barbers (table 15), a fifth barber who was interviewed resided outside the New Orleans study area. The interview study also included 2 Negro male patients who were barbers and 2 white female patients who were beauticians and therefore exposed to chemicals. An analysis of these 9 patients, who were at any time actively occupied as barbers or beauticians, revealed considerable ranges in starting ages in the occupation, years spent in the occupation, and ages at diagnosis of bladder cancer. Besides these 9 patients, 2 of 66 white female patients who were in the incidence rates study only (table 10) listed their husband's occupation as barber.

From November 1, 1960, until the completion of the study, we questioned patients about their use of hair tonics, lotions, and other preparations for the hair and scalp. The data, obtained from the comparatively large group of white males, show no correlation with cancer of the bladder:

Preparations for hair and scalp	Cancer patients	Control patients
Used	42	49
Not used	87	87
No information about use	3	—
Total	132	136

The groups of cancer and control patients did not differ essentially with respect to the types, brands, or the length of time they used these preparations.

We kept a record of the patient's history of outdoor employment, *i.e.*, for 4 or more hours each day. No correlation was found between bladder cancer and outdoor work in any of the race-sex groups. In both the cancer and control groups more males than females and more Negro than white males gave a history of such work, a comparatively large proportion having worked outdoors for more than 30 years.

Specific Exposures to Possible Carcinogens

Table 16 shows the exposures about which the patients were questioned, and the percentages of patients acknowledging these exposures. Some

Table 15.—Observed and Expected numbers of white male patients with cancer of the bladder in occupations selected because of excess in Observed patients, New Orleans 1958–64, incidence rates study

Occupation/code*	Observed No.	Expected No.	S.I.R.†	Indirect‡ age-adjusted incidence rate
Sailors and deckhands/673§	7	0.85	820	134.9
Tinsmiths and coppersmiths/591§	4	0.53	759	124.7
Machinists/544§	4	0.99	406	66.7
Civil engineers/043	3	0.87	346	56.8
Plumbers/574	4	1.24	324	53.2
Structural metal workers/585	1	0.31	324	53.3
Barbers/740	4	1.45	276	45.4
Carpenters/510	7	3.02	231	38.1

*Alphabetical index of occupations and industries, Bureau of the Census, U.S. Department of Commerce, Washington, D.C., 1950 (6).

†The S.I.R. (Standard Incidence Ratio) is the ratio of the observed number of cases to the expected number of cases × 100. For example, the risk of bladder cancer is apparently 8.2 times as large for sailors and deckhands as it is for the total group of white males residing in New Orleans.

‡The indirect age-adjusted incidence rate is obtained by multiplying the crude rate for the entire white male population (16.4) by the S.I.R.

§Statistically significant [5% two-tailed Poisson distribution (7)].

patients promptly recognized and admitted specific exposures, while others offered positive information only while they were discussing their history of occupations. In general, the history of specific exposures is difficult to obtain, and probably some patients were unaware of their past exposures. The interviewer inquired about β-naphthylamine (a known bladder carcinogen), X-radiation, and a number of other suspected carcinogens. Over half the male patients and about five-sixths of the female patients gave no history of exposures to any agent listed in the table. Exposure of white male patients with cancer of the bladder to dusts was excessive at a significant level ($P<0.05$). Fifty-eight of 265 patients with cancer of the bladder were exposed in contrast to 38 of 273 controls, *i.e.*, 21.9 and 13.9 percent, respectively.

The following is an analysis of the types of dust to which the white males were exposed. The percentages below are based on 83 exposures listed for 58 patients with cancer of the bladder and 64 exposures listed for 38 control patients:

Group	Total white males		Percentage of white male patients by type of dust				
	Number	Percent	Asbestos	Chromate	Silica	Arsenic	Other dusts*
Cancer	265	100	2.3	7.2	3.4	0.4	18.1
Control	273	100	2.2	4.0	2.2	0.7	14.3

*Include sawdust, chemical and stone dusts other than those tabulated, and dusts from coal, cotton, sugar, rice, and other grains.

An excess of patients with bladder cancer were exposed to chromates, 19, or 7.2%, in contrast to 11, or 4.0%, for the controls. Since 10 of the 19 exposed patients, 52.7%, were under age 55 when their cancers were diagnosed, in contrast to 19.5% of the total group of white males with cancer of the bladder, a relatively early onset of bladder cancer seemed related to the chromate exposure. However, we could not base firm conclusions on the importance of exposure to chromates when numbers of cases were small and few details were available about forms of exposure.

When the occupations of the 19 cancer patients reporting an exposure to chromate dust were examined, only one was a tinsmith and one a machinist. Hence there is no apparent correlation between cancer patients with an increased risk due to chromate exposure and those in occupations with a high S.I.R. However, other tinsmiths, coppersmiths, and machinists may have been unaware of their exposure to chromate dust.

Alcoholic Beverages

Each patient was asked about the use of wine, beer, and spirits (distilled liquors). Amounts in ounces of each type of beverage were estimated by asking the patient to select the standard beverage containers he used from natural-sized profile drawings of such containers. From the results, present or past use of these beverages was graded as occasional, light, moderate, and heavy. For example, patients who drank spirits were in the "heavy" range if they consumed more than 33 ounces per week; beer drinkers were in the "heavy" range if they consumed more than 216 ounces per week. These ranges were chosen for our convenience in analyzing the data and are not intended as a clinical classification implying specific terms such as "social drinking" or "alcoholism."

Information was also collected about daily drinking, weekend drinking, age at starting use of alcohol, and the duration of its use. We did not find a consistently positive or negative correlation with the use of alcoholic beverages.

Coffee

Several types of coffee are consumed by New Orleans residents. Chicory is frequently added to coffee. Table 17 shows the use of plain, chicory, and dark roast coffees by patients with cancer of the bladder and by control patients.

There was a significant association of bladder cancer with coffee drinking among Negroes ($P = 0.005$). However, when data were broken down by sex, only Negro females showed a significant association ($P<0.025$). There was also a significant correlation of bladder cancer with the amount of coffee consumed by Negro females. None of the above associations was seen among white patients, nor was there an association with types of coffee consumed by any of the four race-sex groups.

TABLE 16.—Patients with bladder cancer and control patients acknowledging specific exposures to possible carcinogens

Group	Total patients in study	Number of patients with specific exposures	Percentages of patients exposed to: Tar	Automotive exhaust fumes	Dyes	Solvents: commercial, industrial	Insecticides	X-radiation	β-Naphthylamine	Arsenic	Dusts
Cancer:											
White male	265	92	6.4	9.8	3.4	4.5	6.0	3.4	0.8	1.1	21.9
Negro male	69	22	4.3	7.2	1.4	5.8	11.6	—	—	1.4	18.8
White female	112	17	—	—	0.9	—	3.6	6.3	—	0.9	4.5
Negro female	47	5	—	—	4.3	4.3	—	4.3	2.1	—	2.1
Control:											
White male	273	84	6.6	9.2	4.4	5.9	7.3	2.9	1.5	3.3	13.9
Negro male	77	32	1.3	10.4	—	9.1	5.2	—	2.6	3.9	26.0
White female	122	16	3.3	—	0.8	—	4.1	1.6	—	1.6	7.4
Negro female	55	3	—	—	—	1.8	1.8	1.8	—	—	3.6

TABLE 17.—Use of coffee

Group	Total		No coffee		Type of coffee: Plain coffee		Chicory coffee*		Dark roast coffee*		Dark roast coffee and chicory coffee*		Unknown	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Cancer:														
White male	265	100	5	1.9	65	24.5	142	53.6	21	7.9	32	12.1	—	—
Negro male	69	100	6	8.7	7	10.1	47	68.1	2	2.9	7	10.2	—	—
White female	112	100	14	12.5	23	20.5	44	39.3	4	3.6	27	24.1	—	—
Negro female	47	100	2	4.3	3	6.4	30	63.8	3	6.4	9	19.1	—	—
Control:														
White male	273	100	10	3.7	69	25.3	140	51.3	7	2.6	43	15.7	4	1.4
Negro male	77	100	16	20.8	2	2.6	41	53.2	3	3.9	15	19.5	—	—
White female	122	100	11	9.0	27	22.2	57	46.7	2	1.6	25	20.5	—	—
Negro female	55	100	13	23.6	2	3.7	32	58.2	—	—	7	12.7	1	1.8

*Many patients in these categories also drank plain coffee.

Proprietary Soft Drinks and Daily Total Consumption of Fluids

Amounts of soft drinks consumed were not correlated with cancer of the bladder.

Table 18 shows total daily ingestion of fluids based on the information elicited. White female controls (44.3%) consumed more than 6 pints of total fluids per day, while only 30.3% of the corresponding patients with cancer of the bladder consumed this amount. This might be accounted for by a higher frequency of diabetics among the control patients. Except for this, the differences between cancer and control patients were minimal.

Foods

The following foods were classified by their customary frequency in diets: Total meat, lean meat, fat meat, other animal fats, poultry, fish, eggs, milk and milk foods, butter, oleomargarine and other vegetable fats, citrus fruits, other fruits, leafy and yellow vegetables, beans and peas, sweet potatoes, white potatoes, breads and other cereal foods, desserts and other sweets, spices and seasonings. The purpose was to identify conspicuous differences in dietary habits between cancer and control patients. All categories of foods were tabulated by their frequency of use, and the data were statistically analyzed. We did not find any strong or consistent correlation with cancer of the bladder.

Use of Tobacco

Table 19 shows the forms of tobacco used by both groups of patients. This table includes both past and present use. When data for all forms of tobacco are combined, there is a significant correlation between bladder cancer and tobacco use among males only ($P<0.025$), but this correlation does not exist when these same data are separated by race or the type of tobacco. Detailed analyses for each form of tobacco with respect to past and present use separately, age at starting, amounts (cigarettes, table 20), and length of use were conducted. Analysis of data on cigarette smoking by the four race-sex groups shows no significant correlation between cigarette smoking and cancer of the bladder, though there are relatively more cancer patients who smoked cigarettes than there are controls who smoked cigarettes in each of the race-sex groups. When data are combined by sex, the association between bladder cancer and cigarette smoking is almost statistically significant for males ($P = 0.06$), but not for females.

Data for cigarette smoking by the number of cigarettes smoked show no meaningful correlation, either with or without considering racial group, though there appears to be an excess of white male cancer patients who reported smoking 10–19 cigarettes per day. In summary, there was a significant correlation between bladder cancer and tobacco use, all forms combined, among males only; however, this correlation did not exist when data were analyzed by type of tobacco used or amounts used.

Unusually Young Patients

A strong, fast-acting environmental carcinogen might be suspected in young patients with cancer of the bladder. Information on 5 patients, age 14–26 years, is shown in table 21. No exposure to known bladder carcinogens emerged, though we did not carry out detailed analysis of the exposures listed in the table.

Two Brothers and Two Married Couples

Information about the 5 patients we interviewed is given in table 22. Results are not reported for birthplace, residence history, and education grade level, because we did not find a pattern in the distribution of these factors. The table does not include the dietary history, which was analyzed but was not informative. Though 4 of the 5 patients gave a history of drinking chicory coffee, the amounts consumed varied widely. An association with chicory coffee had not been found in the analysis of coffee use by the patients with cancer of the bladder (table 17). Other features of the past history of these patients varied.

DISCUSSION

Pathology

In contrast to previous studies on the pathology of bladder cancer which have utilized materials from a single hospital or selected cases submitted to a registry, our study included nearly all cases over a 6-year period from all hospitals in our city, New Orleans. Our histologic classification of

TABLE 18.—Current daily consumption of fluids

Group	Total		Pints of fluid										Unknown amount	
			Less than 2		2–4		5–6		7–8		9 and over			
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Cancer:														
White male	265	100	7	2.6	34	12.8	77	29.1	74	27.9	72	27.2	1	0.4
Negro male	69	100	1	1.4	13	18.8	23	33.4	16	23.2	16	23.2	-	—
White female	112	100	6	5.4	26	23.2	45	40.2	21	18.7	13	11.6	1	0.9
Negro female	47	100	2	4.2	10	21.3	13	27.7	13	27.7	9	19.1	-	—
Control:														
White male	273	100	3	1.0	43	15.8	90	33.0	72	26.4	65	23.8	-	—
Negro male	77	100	1	1.2	11	14.3	28	36.4	16	20.8	21	27.3	-	—
White female	122	100	4	3.2	39	32.0	25	20.5	28	23.0	26	21.3	-	—
Negro female	55	100	-	—	8	14.5	24	43.7	13	23.6	9	16.4	1	1.8

TABLE 19.—Use of tobacco

Group	Total		Type of tobacco use in percent										
	Number	Percent	Never used	Occasional use only	Cigarette only	Cigarette and other	Cigar only	Cigar and other *	Pipe only	Pipe and other †	Chewing tobacco only	Chewing tobacco and use of snuff	Use of snuff only
Cancer:													
White male	265	100	9.8	—	42.6	29.1	3.0	5.3	0.4	1.5	1.1	—	—
Negro male	69	100	4.4	—	47.8	30.4	2.9	7.3	4.4	1.4	—	1.4	—
White female	112	100	67.8	—	30.4	—	—	—	—	0.9	—	—	0.9
Negro female	47	100	48.9	—	38.2	4.3	—	—	4.3	2.1	4.2	—	—
Control:													
White male	273	100	15.4	—	47.6	25.3	2.9	5.5	1.5	1.1	0.3	0.4	—
Negro male	77	100	11.7	—	37.7	31.2	7.8	6.5	3.8	—	—	1.3	—
White female	122	100	68.0	0.8	27.1	0.8	—	—	—	0.8	—	—	2.5
Negro female	55	100	49.1	1.8	30.9	5.5	—	—	5.5	1.8	3.6	—	1.8

* Excludes cigarette smoking.
† Excludes cigarette smoking and cigar smoking.

TABLE 20.—Use of cigarettes

Group	Total		Number of cigarettes per day									
			None		Less than 10		10–19		20 and more		Unknown No.	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Cancer:												
White male	265	100	55	20.8	19	7.2	76	28.7	114	43.0	1	0.3
Negro male	69	100	14	20.3	9	13.0	25	36.3	21	30.4	—	—
White female	112	100	77	68.7	6	5.4	12	10.7	17	15.2	—	—
Negro female	47	100	28	59.6	8	17.0	5	10.6	6	12.8	—	—
Control:												
White male	273	100	71	26.0	21	7.7	46	16.9	133	48.7	2	0.7
Negro male	77	100	24	31.2	18	23.3	16	20.8	19	24.7	—	—
White female	122	100	88	72.1	10	8.2	14	11.5	10	8.2	—	—
Negro female	55	100	35	63.7	10	18.1	6	10.9	4	7.3	—	—

TABLE 21.—Information about unusually young patients with cancer of the bladder

Age/group	Occupation	Specific exposure	History of other bladder disease	Maximum cigarette use per day	Use of coffee (type)
14/white female	Student	Lived near oil refinery, used hair spray, 2 years.	None	None	None.
17/white male	Student	Lived near box factory.	None	10 to 19	Plain.
24/Negro male	Printing, seaman, construction.	Shipyards, inks, cleaning agents, dyes.	None	20 or more	Plain and other kinds.
26/white female	Sales and clerking.	Lived near gas fumes from meat packing plant.	Cystitis, 5 and 2 yrs. previously.	20	None.
26/Negro female	Sewing factory worker.	Cloth dusts	None	Less than 10	Chicory; dark roast.

bladder tumors proved to be workable for the present study. Although it included benign papilloma, we found no lesions which met the criteria for this diagnosis. The distribution of the 3 grades of transitional cell carcinoma in our study (table 2) is generally similar to that found by Mostofi in the American Tumor Registry (*4*), though we classified more tumors as Grade 2 (39 rather than 34%) and fewer as Grade 3 (24 rather than 34%). Mostofi classified 10–12% of his series as squamous cell carcinoma, whereas we found only 4.3%. Of the tumors we classified as transitional cell carcinoma, approximately one-fifth had a few small foci of squamous differentiation in the form of small pearls with little or no keratin. The lesions we diagnosed as squamous cell carcinoma consisted predominantly of keratin, pearl formation, and cells with intercellular bridges. Not infrequently, portions of the squamous cell carcinomas were undifferentiated. We did note the more frequent occurrence of squamous cell carcinoma in Negro patients of both sexes compared to white patients, but do not understand the significance of this observation. In Egypt, with a high relative frequency of bladder cancer and in Mozambique with a high rate, squamous cell carcinoma comprises about 60% of all bladder tumors and this histologic type is associated with

TABLE 22.—Two brothers and two married couples with cancer of the bladder*

Group and age	Interval between onset of disease in patients	Duration of urban residence	Medical history	Occupations, in order of employment, and years employed	Estimated daily use of alcohol and duration of use	Coffee: type, maximum use, duration of use	Daily number of cigarettes, and duration of smoking habit
Two brothers:							
Age 52	32 months	Age 32 to present	Arthritis, age 47	Salesman, 22 yr; oil refinery, 15 yr.	32–48 oz beer, age 16–50.	Plain coffee, 2–3 cups, childhood to present.	10–19, age 16 to present.
Age 57a		Age 47 to present	(None listed)	Carpentry, shipyard, fireman, 13 yr; farming, 20 yr	(None listed)	Chicory coffee, 12–14 cups, childhood to present.	25–30, age 12 to present.
Married couple:							
Wife, age 69	3 months	"Life"	Pelvic X radiation,† age 42; urethral polyps, age 59.	Stenographer, 3 yr; housewife, remainder.	3–5 oz whiskey, age 50 to present.	Chicory coffee, 1–2 cups, childhood to present.	(None listed).
Husband, age 78		(Patient refused to be interviewed)					
Married couple:							
Husband, age 64	39 months	Age 2 to present.	(None listed)	Grocery store, 2 yr; bartender, 6 yr; seaman in engine room, 31 yr.	96–112 oz beer, age 18–62	Chicory coffee, 8–10 cups, childhood to present.	30–60, age 14 to present.
Wife, age 68		"Life"	Hysterectomy,† age 39.	Grill and restaurant, 16 yr; grocery store, 14 yr.	(None listed)	Chicory coffee, 10 cups, childhood to present.	20 and over, age 35 to present.

*All were white patients; cancer of the bladder developed first in the younger patient of each pair.
†Indication for treatment is not known.

schistosomiasis (*8*, *9*). We saw no evidence of schistosomiasis in any of the tissues from New Orleans patients. Most bladder tumors in American Indians are squamous cell carcinoma, even though the Indians have a low frequency ratio of bladder cancer and are not exposed to schistosomiasis (*10*).

Incidence Rates

The incidence rates of bladder cancer in our survey from 1958–64 were nearly 50% lower than those in the 1947 survey by Dorn and Cutler, except that the rate for Negro males had increased by about 50%. There are at least two possible explanations. Cancer of the bladder may actually have increased in the Negro males because of increased exposure of this group to carcinogens, or more cases may have been detected in 1958–64 than in 1947. We have no data that favor either hypothesis. With regard to the apparent decrease in bladder cancer rates in the other groups, we did find marked yearly variations in cancer rates and 1947 could have been a comparatively "high rate year." Our 6-year study allowed more time for verification of dates of diagnosis, residence at time of diagnosis, and histologic diagnoses than the 1-year study in 1947 and we excluded some cases that might have been retained in a 1-year survey; however, these cases are not enough to account for the observed decrease. The possibility remains that there has been a real decrease in bladder cancer in New Orleans from 1947 to 1958–64 and that this resulted from the removal of some carcinogen from the environment.

Interview Study

We included questions about factors suspected of contributing to the causation of bladder cancer (*11–20*). A study involving large numbers of variables may be expected to show correlations at conventional significance levels purely by chance. We have tried to avoid placing undue emphasis on correlations that may not be related to etiology.

From the history, there were statistically significant correlations with cystitis for both white and Negro males and with prostatic diseases for white males only. In addition to the role that may be played by chance, several features of the data we obtained make it difficult to interpret whether the above factors may be related to bladder cancer. The numbers of patients with the associated factors were small. Also, we could not specifically exclude the possibility that patients who suffer from urinary tract complaints because of cancer in the bladder are more likely than other patients to recall and report cystitis and prostatic disease. The prostatic diseases were diagnosed mainly in the 3-year period before cancer of the bladder was diagnosed. Possibly not all these patients knew they had cancer of the bladder and some may have mistakenly thought they had a prostatic disease only.

The Surgeon General's Report on Smoking and Health (*21*) reviews the results of 4 retrospective and 7 prospective studies with regard to smoking. The conclusion of the report was that an association does exist between cigarette smoking and urinary bladder cancer in males, but the association is not sufficient to support a judgment on the causal significance of smoking. The 4 retrospective studies found a significant association between cigarette smoking and urinary bladder cancer in males. Three studies also included data on female cigarette smokers, but only 1 found a significant relationship. The results of additional studies since the Surgeon General's Report are discussed in a later review (*22*). However, they left the conclusion of the original report unchanged. Hence, though the results of our study of smoking history do not duplicate those of the retrospective studies cited, they lie in the same direction and do not conflict with them.

Exposure to dusts was at a significant level for the group of white males with bladder cancer. The excess was mainly for exposure to chromates, not specified in further detail, and only a small total number of patients were so exposed. Investigators who have studied cancer of the bladder have not established an increased risk among workers in the chromate industry, who are known, however, to have an excess of cancers of the respiratory tract (*13*).

A few correlations with occupations were noted. Statistical limitations are apparent in epidemiologic studies such as ours that include relatively small numbers of cases, and these limitations necessarily affect our interpretation of the results. We interviewed 493 patients with cancer and 527 con-

trol patients. These groups were further subdivided by the 4 population groups, white and Negro, males and females. The problems in connection with numbers of cases may be shown in the largest group and thus the group that is most stable from a statistical viewpoint. This group consisted of 265 white males with bladder cancer and 273 controls. There were many individual categories of occupations so that only small numbers of patients were included in each category. The ratios of occupations of cancer patients to those of control patients were: mechanics and repairmen 22 : 13, welders 8 : 4, tinsmiths and coppersmiths 6 : 1, barbers 5 : 2, civil engineers and teachers 4 : 1 each, and structural metal workers and bartenders 3 : 1 each. The comparatively small differences between numbers of cancer and control patients may have been due to chance only. When the occupations are grouped in broad categories to afford comparatively large numbers for analysis, we lose potentially valuable information about specific exposures in particular occupations. For instance, the category "craftsmen, foremen and kindred workers" includes 37 diverse occupations of the patients in the study. Analysis of the data on industries is handicapped by the same problems and raises similar questions. One of the broad categories, "personal services" for example, includes six diverse categories, *i.e.*, private households, hotels and lodging places, laundering cleaning and dyeing services, dressmaking shops, shoe repair shops, and miscellaneous services, a grouping which results in obscuring all information about specific exposures.

In addition, our confidence in the analysis and interpretation of ratios of "Observed" to "Expected" cases of white male patients in certain occupations, is lessened by the following: 1) The number of "Observed" cases was based on the present or last position held by the patients. Since the majority of patients were over age 65, it is likely that some of the persons in "Observed" occupations were actually retired, that is, not classified in an occupation by the census. Thus the number of patients reported as "Observed" is probably too high. 2) In most instances "Expected" numbers are very small. Thus an excess of one or two persons "Observed" in an occupation results in a high S.I.R. and a correspondingly high age-adjusted incidence rate for this occupation. However, on this basis the excess may be an artifact produced by the conditions under which the study was carried out.

Our analysis of the data obtained from special groups of patients, *i.e.*, young patients, two brothers, two married couples, and patients who used proprietary preparations for the hair or scalp, did not reveal associations we could consider significant.

We are left with essentially inconclusive results in a study where the methods raise doubts about the significance of the few factors that were found weakly associated with cancer of the bladder. Provided we have included questions about the significant factors, a genuinely strong association with bladder cancer should have become apparent in a study such as ours. We did not find any such strong association. Possibly, a carcinogen infrequently encountered in the environment was not identified because it was responsible for relatively few bladder cancers. A further difficulty is that our study and similar studies fail to provide sound means of assessing what might be the effects of two or more factors that produce bladder cancer only when acting together.

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Chemical Mutagens in the Human Environment

CHEMICAL mutagens in the environment are a hazard which has not so far been systematically explored. Although certain mutagens—notably ionizing radiations and alkylating agents—have been studied in detail in bacteria[1,2] and *Drosophila*[3,4], their relevance to man is not always apparent. M. Legator (personal communication) has considered the influence of host-mediated drug effects by studying mutagenicity in bacteria in a mammalian environment. Lining[5], in discussing testing for mutagenicity, stated: "The use of mammals in such tests would be both expensive and time-consuming, and might slow down even more the development of new drugs", but Bateman[6] considered the use of mammals in tests of mutagenicity to be practical and more meaningful than studies with other systems in the context of human hazards. We present here preliminary data on the feasibility of mutagenicity testing in mice based on screening a wide variety of environmental pollutants, including carcinogens, air and water pollutants, pharmaceuticals, food additives and pesticides.

In the dominant lethal test, treated males are sequentially mated with groups of females. The females are subsequently dissected at mid-term of pregnancy to evaluate the incidence of dominant lethal mutations. Such effects conveniently reflect mutagenic activity and represent foetuses killed *in utero* by mutations directly induced in male germ cells. The possibility of systemic drug effects in females is thus excluded.

In general, LD_5 doses were selected for testing. Where strong anti-fertility effects were evidenced at these doses, testing was repeated at lower levels. Test materials were prepared freshly and usually injected intraperitoneally in tricaprylin solution or suspension in 0·1 ml. volume. Male Swiss mice (*CD*-1), 8–10 weeks old, were dosed in minimal groups of five. Groups of ten male mice, injected with tricaprylin, served as controls for concurrent testing of seven–nine samples. Within 2 h of injection, each male was caged with three virgin females, 8–10 weeks old, for 7 days. After 7 days, the females were replaced with fresh virgin animals. This breeding schedule was maintained for 8 consecutive weeks—the duration of the spermatogenic cycle in the mouse. All females were autopsied 13 days from mid-week of their mating; pregnancy thus ranged from 9–15 days. In general, fertilizations were maximal during the middle portion of each mating week. At autopsy, animals were scored for total implantations, early deaths—appearing as small black deciduomata—and late deaths. Corpora lutea were counted under a low-power dissecting microscope.

A mutagenic index (M.I.)[7], reflecting the incidence of dominant lethal mutations in an experimental group of animals, is conventionally calculated as follows

$$\text{M.I.} = \frac{\text{deciduomata} + \text{late deaths}}{\text{total implantations}} \times 100$$

This index, however, is limited in that it is perforce increased by reduction in total implantations alone; additionally, it reflects neither anti-fertility effects nor the distribution of deciduomata in affected animals.

Results are summarized in Table 1, where all effects are listed for the third mating week, with the exception of aflatoxin (week 1) and methyl methane sulphonate (week 2). The average composite control value of M.I., based on 150 females, for the third week was 1·0 per cent, with a range of 0 per cent to 3 per cent. Control M.I. values of 3–10 per cent have been reported for other strains of mice[7,8]. The incidence of late deaths was extremely low; one late death appeared in each of five out of a total of forty-five compounds tested, for the third week of mating. In control females for the third week, three late deaths were seen. Mutagenic activity was, in general, maximal in weeks 2 and 3.

Table 1. MUTAGENIC EFFECTS IN MICE OF ENVIRONMENTAL POLLUTANTS: CARCINOGENS, AIR AND WATER POLLUTANTS, PHARMACEUTICALS, FOOD ADDITIVES AND PESTICIDES

Compound	Dose (mg/kg)	No. females bred	No. pregnant (percentage)	Total No. implants	Av. No. implants/mouse	Total No. deciduomata	Mutagenic index
(*A*) Controls	—	150	116 (77)	1,434	12·4	12	1
(*B*) Carcinogens:							
Aflatoxin	68*	9	7 (78)	73	10·4	8	11
Benzanthrone	1,000	12	10 (93)	125	12·5	0	1
Benzo[*a*]pyrene	750	18	7 (39)	53	7·6	5	11‡
Butter yellow	216	21	14 (67)	170	12·1	2	1
Cumene hydroperoxide	34	15	13 (87)	167	12·8	5	3
1,2,3,4-Diepoxybutane	7	21	17 (81)	206	12·1	4	2‡
Dimethylhydrazine	25	21	14 (67)	165	11·8	2	1
Dimethylnitrosoamine	8	15	11 (73)	132	12·0	3	2
Hydrazine	42†	12	12 (100)	135	11·3	1	1
3-Methylcholanthrene	100	15	12 (80)	113	9·4	2	2
4-Nitroquinoline-1-oxide	5	21	17 (81)	204	12·0	3	2
Urethan	1,000	15	13 (87)	156	12·0	2	2‡
(*C*) Organic extracts of atmospheric particulate pollutants:							
Boston 1966	333	21	16 (76)	200	12·5	4	2
Acid fraction, New York 1967	333	21	13 (62)	159	12·2	0	0
Basic fraction, New York 1967	666	18	14 (78)	163	11·6	2	1
Insoluble fraction, New York 1967	333	21	18 (86)	222	12·3	5	2
(*D*) Foods and food additives:							
Butylated hydroxytoluene	1,000	9	4 (44)	46	11·5	0	0
Caffeine	168	21	12 (57)	124	10·3	0	0
Theobromine	380	18	12 (67)	182	11·0	2	2
Theophylline	380	12	11 (92)	119	10·8	3	3‡
(*E*) Organic extracts of finished drinking water:							
Maine 1961	333	21	9 (43)	95	10·6	1	1
Yonkers 1961	699	21	11 (52)	139	12·6	1	1
(*F*) Pesticides:							
Captan	9	21	20 (95)	210	10·5	2	1
Captan	500¶	18	15 (83)	171	11·4	1	1
DDT	105	18	13 (72)	135	10·4	4	3
Maleic hydrazide	500	15	11 (73)	121	11·0	1	1
(*G*) Pharmaceuticals:							
Aminopterin	10	12	9 (75)	88	9·8	0	0
Azaribine	1,000¶	21	14 (67)	151	10·8	1	1
5-Bromodeoxyuridine	500	27	21 (78)	248	11·8	1	0
Chloramphenicol	333	21	14 (67)	128	9·1	2	2
Chlorpromazine	8·3†	27	20 (74)	237	11·9	1	0
Griseofulvin	750	18	14 (78)	175	12·5	3	2
Hydroxyurea	500†	21	14 (67)	160	11·4	0	0
5-Iododeoxyuridine	250	21	16 (76)	192	12·0	2	1
METEPA	40	21	16 (76)	145	9·1	39	27
Methyl methane sulphonate	50	24	19 (79)	164	8·6	47	29
TEPA	7	18	13 (72)	115	8·8	44	38
THIO-TEPA	5†	18	15 (83)	114	7·6	21	18
Triethylene melamine	0·2	21	15 (71)	137	9·1	36	26
(*H*) Miscellaneous:							
Acriflavine	10·6	21	14 (67)	172	12·3	0	0
Acrolein	1·5	15	12 (80)	152	12·7	7	5
Dimethyl sulphate	23	18	14 (78)	150	10·7	1	1
Formaldehyde	20	24	19 (79)	233	12·3	4	2
Methylhydroxylamine	140†	21	12 (57)	133	11·1	0	0
Pinacyanole	1·9	12	8 (67)	99	12·4	1	1

* Dimethyl sulphoxide suspension. † Aqueous solution. ‡ One late death occurred in each of these cases. ¶ Oral administration.

Among the carcinogens tested, aflatoxin and benzo[*a*]pyrene were mutagenic. The aflatoxin sample—a mixture of aflatoxins B_1 and G_1—showed a singular response in that activity commencing in week 3 persisted through week 5. Work on this substance, in collaboration with Professor G. T. Wogan, is continuing. M.I. values of other carcinogens which were tested were at control levels throughout the 8-week breeding cycle. The ethyleneimine alkylating agents, TEPA, METEPA, THIO-TEPA, and triethylene melamine were all strongly mutagenic in weeks 2 and 3. The effects of methyl methane sulphonate, mutagenic as previously reported[7], were maximal in week 2, and declined to control levels by week 3. Organic extracts of atmospheric particulate pollutants and of finished drinking water displayed control M.I. levels. The latter samples consisted of a reconstituted mixture of ethanol and chloroform adsorbate extracts in an aqueous solvent. Butylated hydroxytoluene and the three xanthines tested, including caffeine, showed control M.I. values. Caffeine breaks chromosomes in human cell cultures[8], is mutagenic in bacteria[10], and also in mice for whom the induction of dominant lethal mutants has been claimed by Dr W. Ostertag (personal communication) on the basis of indirect evidence of pre-implantation loss following chronic administration in drinking water. Inconsistent results, however, have been reported in *Drosophila*[11,12]. The pesticides tested yielded M.I. values in the control range. Captan was administered both intraperitoneally and, at higher doses, orally.

Sensitivity of mice to this representative sampling of mutagenic agents is usually restricted to post-meiotic stages of spermatogenesis, consistent with an action on non-replicating DNA. The present testing schedule might therefore be simplified with little sacrifice of screening efficacy. Primary screening might be based on administration of a single LD_5 dose to five male mice which would then be mated only during the second and third weeks following injection. The number of animals required would thus be greatly reduced. Controls apart, only thirty females and five males would be needed for each drug. Positive findings revealed by this screen could be further explored. Testing could then cover the whole of the spermatogenic cycle, more animals, alternative routes of short and long-term drug administration and a wider dose range.

The diversity of potential chemical mutagens to which man is exposed urges the development of practical screening procedures. The dominant lethal test seems suitable, especially because 80 per cent of gene mutations in man are attributable to dominant autosomal traits. The test, however, provides no information on point or recessive mutations nor on non-mutagenic sperm damage which may, nevertheless, modify nucleic acid [illegible]. Modification of the test procedures to evaluate the [illegible] of chronic low level exposure to environmental mutagens either singly or in combination, is now in progress. Possibly, differences between the reproductive cycles of mouse and man may, additionally, perturb direct extra-

polation of risk. Primates aside, however, lower mammals provide the simplest means of relating mutagenic effects to human genetic hazard.

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The Failure of Caffeine to Induce Mutagenic Effects or to Synergize the Effects of Known Mutagens in Mice

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Abstract—Caffeine administered to male mice acutely or for 8 wk prior to mating produced no mutagenic effect in the dominant lethal assay, as measured directly by increased early foetal deaths or indirectly by increased pre-implantation losses. Furthermore, neither acute nor 8-wk administration of caffeine synergized the effects of the known mutagens, methyl methanesulphonate, TEPA or X-rays.

INTRODUCTION

High concentrations of caffeine are mutagenic in *Escherichia coli* (Demerec, Bertani & Flint, 1951; Gezelius & Fries, 1952; Novick, 1956). The literature on the genetic effects of caffeine in Drosophila has recently been reviewed and found to be equivocal (Bateman, 1969). Caffeine inhibits DNA repair in *E. coli* (Lieb, 1961; Witkin, 1958), and in cells from the mouse (Rauth, 1967) and man (Wragg, Carr & Ross, 1967) *in vitro*. These findings are of considerable interest and raise the possibility that caffeine may be a direct or synergistic mutagenic hazard to man. Although the genetic apparatus is fundamentally the same in all biological systems, the differences between bacteria and man are so great that direct extrapolation of bacterial data to man is not valid. These differences relate to the role of factors such as cell uptake and transport and microsomal-enzyme metabolism. It is now generally accepted, therefore, that data from sub-mammalian species and from *in vitro* mammalian systems have little direct relevance to man (Epstein, 1969a), particularly with reference to mutagenic and other hazards.

The literature on *in vivo* testing of caffeine in mammals has been authoritatively reviewed by Röhrborn (1968), who emphasized that no definitive data had demonstrated any mutagenic effect of caffeine. Claims to the contrary (Kuhlmann, Fromme, Heege & Ostertag, 1968; Ostertag, Duisberg & Stürmann, 1965) were shown to be founded on invalid or inferential data. More recently, long-term treatment with caffeine has failed to induce aberrations in the meiotic chromosomes of male mice (Adler & Röhrborn, 1969). High doses of caffeine administered parenterally to male mice failed to induce dominant lethal mutations (Adler, 1969).

We present here data on the testing of caffeine alone for the induction of dominant lethal mutations in mice, and of caffeine in combination with various known mutagens for the

induction of synergistic mutagenic effects. Since toxicologists tend to be relatively unfamiliar with the dominant lethal assay, some preliminary comments on its background and theoretical implications and on its recent practical applications are appropriate.

Principles of the dominant lethal assay in mice

Dominant lethal mutants are convenient indicators of major genetic damage and have been used in mammals for measuring the effects of X-rays (Bateman, 1958) and, more recently, of chemical mutagens (Bateman, 1964 & 1966; Cattanach, Pollard & Isaacson, 1968; Ehling, Cumming & Malling, 1968; Epstein & Shafner, 1968; Generoso, 1969; Röhrborn, 1968). Data on the induction of dominant lethal mutants in mammals may be appropriately extrapolated to man, especially as most recognizable human mutations are due to dominant autosomal traits (United Nations Scientific Committee on the Effects of Atomic Radiation, 1966). The genetic basis for dominant lethality is the induction of chromosomal damage and rearrangements, such as translocations, resulting in non-viable zygotes. Evidence for zygote lethality induced in mammals by X-rays and by chemical mutagens, respectively, has been obtained embryologically (Epstein, Joshi, Arnold, Page & Bishop, 1970c; Hertwig, 1940; Snell, Bodenstein & Hollander, 1934; Snell & Picken, 1935) and cytogenetically (Bateman, 1969; Generoso, 1969; Russel & Russel, 1954). Additional evidence for the genetic basis of dominant lethality is derived from its associated induction of sterility and inheritable semi-sterility in the F_1 progeny of males exposed to X-irradiation (Koller & Auerbach, 1941; Snell *et al.* 1934) and to chemical mutagens (Cattanach, 1964; Epstein, Bass, Arnold, Bishop, Joshi & Adler, 1970b; Falconer, Slizynski & Auerbach, 1952); translocations have been demonstrated cytologically in such semi-sterile lines in mice (Cattanach *et al.* 1968; Epstein *et. al* 1970b; Koller, 1944; Slizynski, 1952), in hamsters (K. S. Lavappa and G. Yerganian, personal communication in 1969) and more recently in foetal mice (E. Schleiermacher, personal communication in 1969).

The induction of dominant lethal mutations in animals can be assayed, with a high degree of sensitivity and practicality, following acute, subacute or long-term administration of test materials, either orally or by the respiratory or any other parenteral route. It is feasible, therefore, to integrate such tests into routine toxicological practice (Epstein, 1969b). After administration of the drug, male rodents are mated sequentially with groups of untreated females over the duration of the spermatogenic cycle, which in mice lasts approximately 42 days and comprises the following stages: spermatogonial mitoses, 6 days; spermatocytes, 14 days; spermatids, 9 days; testicular sperm, 5·5 days; epididymal sperm, 7·5 days (Bateman, 1958). Thus, matings with 3 wk of a single administration of a drug represent samplings of sperm exposed during post-meiotic stages, and matings from 4–5 wk later represent samplings of sperm exposed during pre-meiotic and stem-cell stages.

The classical form of the dominant lethal assay involves autopsy of females approximately 13 days after timed matings, as determined by vaginal plugs in mice and vaginal cytology in rats, and the counting of corpora lutea and of total implants (comprising living foetuses, late foetal deaths and early foetal deaths). The test can be considerably modified and simplified, and hence made more suitable for routine practice, by sacrificing the females at a fixed time, e.g. in mice, 13 days from the mid-week of their caging and presumptive mating (Epstein & Shafner, 1968). Additionally, this allows the determination of effects of drugs on pregnancy rates. Similarly, corpora lutea counts, which are notoriously difficult, laborious and inaccurate in mice and afford a measure of total fertilized zygotes, can be omitted and the numbers of total implants in the test animals can be related to those in controls, thus

affording a simple measure of pre-implantation losses. Using such modified procedures, together with computerized data handling, large numbers of compounds can be simply and rapidly tested for mutagenic activity (Epstein & Shafner, 1968). Röhrborn & Berrang (1967) have described a modified form of the dominant lethal assay in female mice, in which both pre-meiotic and post-meiotic germinal stages can be tested

Dominant lethal mutations are measured directly by enumeration of early foetal deaths and indirectly by pre-implantation losses, as indicated by the total number of implants in test animals compared with controls. Results are best expressed as early foetal deaths/pregnant female, rather than by the more conventional mutagenic index (early foetal deaths × 100/total implants), as the latter index can be markedly altered by variations in the total numbers of implants (Epstein, Arnold, Steinberg, Mackintosh, Shafner & Bishop, 1970a). Pre-implantation losses offer a presumptive index of mutagenic effects, but no precise parallel can be drawn between pre-implantation losses and early foetal deaths. These should be regarded as concomitant and not alternate parameters. Furthermore, the use of the mutagenic index presupposes that the number of early deaths is proportional to the number of implants, regardless of pre-implantation losses. Such a supposition assumes that absolute numbers of early deaths are lower in animals with reduced numbers of total implants, but this is not so (Epstein *et al.*, 1970a). Finally, an additional disadvantage of using such ratios as measures of mutagenic effect is that their variability is high, as both numerator and denominator are contributory, and estimates of standard deviation are therefore complex.

Pre-implantation losses, early foetal deaths, sterility and semi-sterility constitute a spectrum of adverse genetic effects of which early foetal deaths clearly afford the most convenient and quantitatively unequivocal parameter of mutagenicity.

Using these techniques, a wide range of environmental chemicals, including pesticides, food additives, drugs and air and water pollutants, have been tested for mutagenicity in mice (Epstein & Shafner, 1968; S. S. Epstein, unpublished data, 1969). Additionally, detailed dose-response studies with the aziridine alkylating agents, tris(1-aziridinyl)phosphine oxide (TEPA) and tris[1,2-(2-methylaziridinyl)] phosphine oxide (METEPA), which have been used in the treatment of neoplastic and non-neoplastic diseases and as chemosterilants, have revealed mutagenic thresholds in the region of 0·04 mg/kg and 1·4 mg/kg, respectively, in mice given a single parenteral dose (Epstein *et al.* 1970a).

EXPERIMENTAL

Animals. The experiments were carried out on Swiss mice of the ICR/Ha strain obtained from The Charles River Breeding Laboratories, Inc., Wilmington, Mass. Males were 8–10 wk old at the start of the experiment and females were 8–10 wk old at mating.

Materials. Caffeine (obtained from Eastman Organic Chemicals, Rochester, N.Y.), methyl methanesulphonate (from K & K Laboratories) and TEPA (also from K & K Laboratories) were freshly dissolved in tricaprylin or distilled water for intraperitoneal injection. Concentrations were adjusted to provide the required dose in an injection volume of 0·1 ml. For the 8-wk treatment caffeine was dissolved in the drinking water, which was freshly prepared daily.

Experimental design and conduct

Tests on caffeine alone. These studies involved both acute and repeated administration of caffeine (Table 1). In the acute studies, caffeine in tricaprylin or distilled water was injected

Table 1. *Dose schedule for and toxicity of acute and 8-wk caffeine administration*

Experiment no.	Duration of mating (wk)	Solvent	Caffeine administration: Single intraperitoneal dose (mg/kg)	Caffeine administration: For 8 wk: Concn in drinking water (%)	Initial no. of males exposed	No. of male deaths
1	3	Tricaprylin	0	—	10	0
			168		9	0
2	3	Tricaprylin	0	—	10	0
			214		5	0
			240		5	0
3	8	Tricaprylin	0	—	10	0
			168		7	0
			192		7	1
4	8	Tricaprylin	0	—	5	0
			178*		20	2
5	8	Water	0		5	0
			200		10	0
6	8	Water	—	0 (control)	10	0
				0·1	10	0

* Two intraperitoneal injections given 24 hr apart.

intraperitoneally into male mice. Total dosage ranged from 168 to 240 mg/kg. The 175-mg/kg level was administered twice at 24-hr intervals. Males injected with solvent alone served as concurrent controls. Following injection, each male was caged for 1 wk with three virgin females which were replaced weekly by fresh animals for a total of 3-8 wk. Wk 1-3 and 4-8 after injection represented samplings of sperm exposed to caffeine in post- and premeiotic phases of development, respectively. All females were autopsied 13 days after the mid-week of their caging and presumptive mating, so that these untimed pregnancies ranged in duration from 9 to 15 days. Each female was scored for pregnancy and for the total number of uterine implants, comprising living implants, late foetal deaths and early foetal deaths. Corpora lutea were not counted.

In the second study, caffeine was administered to male mice at a concentration of 0·1% in the drinking water for 8 wk. At this caffeine level, the average estimated daily water consumption/mouse was approximately 6·4 ml, compared with the normal water consumption of control mice of approximately 8 ml/day. Consequently, controls were limited to the volume of water consumed by the test group on the preceding day. After 8 wk, the caffeine regimen was discontinued and each test and control male was caged with three virgin females each week for 8 successive weeks. The subsequent procedure during mating and autopsy was the same as that in the acute studies.

Tests on caffeine in combination with known mutagens. These studies were based on the acute administration of known mutagens (methyl methanesulphonate and TEPA) alone or in combination with caffeine administered either in single or repeated doses (Table 2). Each drug was administered by intraperitoneal injection to male mice. Mice treated with a mutagen alone were injected also with solvent instead of with caffeine. In acute experiments with X-rays, mice were confined after treatment with caffeine and irradiated from an aluminium-filtered cobalt source. The total dosage of caffeine given in these acute experiments ranged from 168 to 200 mg/kg. The 175 mg/kg dose was administered twice at 24-hr intervals before irradiation. Subsequent procedures during mating and autopsy were as described earlier.

In the prolonged studies, caffeine was administered to male mice for 8 wk in drinking water, at concentrations ranging from 0·05 to 0·4% (Table 2). At caffeine concentrations in drinking water greater than 0·1%, the average daily water intake was lower than the normal intake of about 8 ml/day. After 8 wk the caffeine regimen was discontinued and test and control mice were irradiated as in the acute experiments. Each irradiated male was then caged with three untreated virgin females each week for a total of 8 successive weeks. Subsequent procedures during mating and autopsy were as described earlier.

RESULTS

Tests on caffeine alone

Toxicity. Caffeine was not toxic to male mice at the doses tested. Mortality was sporadic and was not dose-related (Table 1).

Effect on pregnancy rates. In the acute studies, there was no systematic variation in pregnancy rates with time in females mated with control males treated with either tricaprylin or distilled water (Table 3). Data from separate weeks were therefore combined for computational purposes. For the combined tricaprylin controls the mean weekly pregnancy rate for all experiments was 72% and the standard deviation was 9·2%, according well with the expected binomial standard deviation of 8·8%. For corresponding weekly test groups

TABLE 2. *Dose schedule for and toxicity of acute and 8-wk caffeine administration in combination with single doses of known mutagens*

Experiment no.	Duration of mating (wks)	Mutagen treatment: Mutagen (and solvent)	Mutagen treatment: Dosage (mg/kg* or r.†)	Caffeine administration: Single intraperitoneal dose (mg/kg)	Caffeine administration: For 8 wk: Concn in drinking water (%)	Initial no. of males exposed	No. of male deaths
1	8	MMS (triacetin)	50	0	—	9	1
			50	168	—	7	0
			50	192	—	4	0
2	8	TEPA (water)	0·312	0	—	5	0
			0·312	200	—	10	0
			1·250	0	—	5	0
			1·250	200	—	10	0
3	8	X-ray	50	0	—	5	0
			50	200	—	10	0
			200	0	—	5	0
			200	200	—	10	0
4	4	X-ray	200	0	—	9	0
			200	168	—	9	0
5	8	X-ray	250	0	—	16	1
			250	175‡	—	20	1
6	8	X-ray	200	—	0	10	0
			200	—	0·05	9	0
			200	—	0·1	10	0
			200	—	0·2	9	0
7	8	X-ray	200	—	0	20	0
			200	—	0·1	20	0
			200	—	0·2	19	1
			200	—	0·4	15	1
			50	—	0	20	0
			50	—	0·1	20	1
			50	—	0·2	19	1
			50	—	0·4	15	0

MMS = methyl methanesulphonate

* Single intraperitoneal injection.
† Single exposure.
‡ Two intraperitoneal injections given 24 hr apart.

Table [illegible]. *Percentage pregnancy following acute and 8-wk caffeine administration*

Time of mating (wk after treatment)	Experiment no.	1		2			3			4		5		6	
	Treatment	Tricaprylin (control)	Caffeine (168 mg/kg)	Tricaprylin (control)	Caffeine (mg/kg) [illegible]	Caffeine (mg/kg) 240	Tricaprylin (control)	Caffeine (mg/kg) 168	Caffeine (mg/kg) 192	Tricaprylin (control)	Caffeine (175 mg/kg × 2)	Water (control)	Caffeine (200 mg/kg)	Control	Caffeine in drinking water (0·1 %)
		Incidence of pregnancy (%) in													
1		80	26	60	20	60	70	33	48	80	27	53	13	67	47
2		73	53	72	47	[illegible]	83	33	61	87	44	87	13	87	53
3		70	59	67	80	67	67	27	37	87	69	67	57	87	53
4							73	62	67	50	63	80	47	77	57
5							73	62	78	75	56	80	60	83	53
6							50	33	62	80	67	67	60	57	47
7							60	67	72	73	61	87	80	66	53
8							60	27	72	67	43	60	73	67	50
Unweighted overall mean		74·3	46·[illegible]	[illegible]	[illegible]	[illegible]	67·0	50·5	62·4	78·5	[illegible]	73·0	49·1	74·0	51·6
Unweighted standard deviation		5·1	17·[illegible]	[illegible]	30·1	3·1	[illegible]	14·8	12·0	7·0	15·0	12·7	27·5	11·2	3·4

each of 30 females, 95% of the observations should, in the absence of drug-induced effects, fall within the range of 72 ± 18·8% pregnant. The incidence of pregnancy rates below these limits in the test groups was non-systematic, except that rates for the first 2 mating weeks were generally reduced. Mean pregnancy rates for the whole test period were always lower for treated animals than for concurrent controls (Fig. 1). There was thus a highly significant

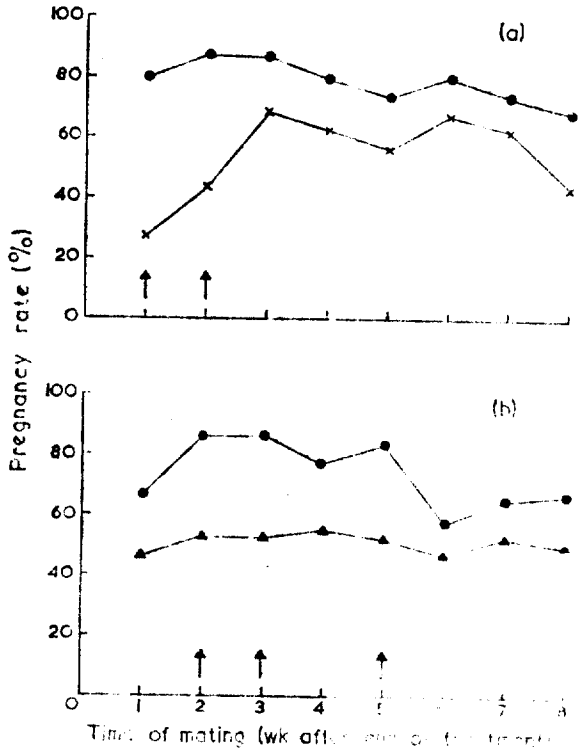

Fig. 1. Effect of caffeine on pregnancy rates in mice following [illegible] the levels [illegible] indicate statistically significant differences ($P < 0·05$) between values for controls (●) and for animals mated with males given two intraperitoneal injections of 175 mg caffeine/kg 24 hr apart [illegible] caffeine in the drinking water for 8 wk prior to mating (▲).

reduction in the overall incidence of pregnancies in females mated with test males compared with females mated with control males ($\chi_1^2 = 7$).

In the 8-wk study the control group showed wide but non-systematic variations in pregnancy rates (Table 3). The mean percentage pregnancy and standard deviation were 74 and 11·2%, respectively. For groups of 30 females, the 95% confidence intervals were therefore 71 ± 22·9%. The pregnancy rate for the test group for each week was below that of the control level, falling along the lower limit of the control range and with the greatest variations from the controls occurring during the first 5 wk (Fig. 1). Since [illegible] each week the pregnancy rate with caffeine-treated mice was lower than with the controls, it is clear that treatment of males with caffeine definitely reduces pregnancy rates (N_1^2 [illegible]).

Effect on total implants and early foetal deaths. Total implants, including living foetuses and early foetal deaths, were scored for each pregnant mouse and the means were determined weekly for all pregnant females in each group (Tables 4, 8, 5). Analysis of variance

Table 4. [illegible] pregnancy following acute and [illegible] caffeine administration

Time of [illegible] (wk [illegible] treat[illegible])	Experimental [illegible]	Mean total implants/pregnancy in													
		1			2		3			4		5		6	
					Caffeine (mg/kg)		Tricaprylin (control)	Caffeine (mg/kg)		Tricaprylin (control)	Caffeine (175 mg/kg 2)	Water (control)	Caffeine (200 mg/kg)	Control	Caffeine in drinking water [illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	240		168	182						
[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	11·1	12·6	12·1	12·6	[illegible]	11·9	13·5	12·0	12·2	[illegible]
[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	10·5	12·2	[illegible]	13·9	[illegible]	11·7	11·8	14·3	11·5	[illegible]
[illegible]				[illegible]	[illegible]	12·[illegible]	11·5	[illegible]	11·9	[illegible]	[illegible]	12·8	12·2	11·3	[illegible]
[illegible]							10·[illegible]	10·9	11·5	[illegible]	12·3	12·1	11·6	11·8	[illegible]
[illegible]							10·8	10·3	10·7	[illegible]	12·1	13·0	11·9	12·4	11·8
[illegible]							12·4	11·3	12·0	[illegible]	13·0	10·4	12·[illegible]	11·9	[illegible]
[illegible]							11·1	[illegible]	12·0	[illegible]	12·4	12·[illegible]	12·1	11·5	[illegible]
[illegible]							12·3	12·8	[illegible]	12·2	12·3	12·3	11·8	11·0	10·8
Unweighted overall mean		11·7	11·8	11·3	11·1	11·2	11·7	11·7	11·5	[illegible]	12·3	12·3	12·3	11·7	11·7
Unweighted standard deviation of mean		[illegible]	0·57	0·29	0·51	0·81	0·85	1·09	0·74	0·66	0·43	0·97	0·85	4·7	4·3

Table 5. Mean [illegible] fetal deaths/pregnancy following acute and 18-wk caffeine administration

Time of [illegible] (wk [illegible] treat[illegible])	Experimental [illegible]	Mean early fetal deaths/pregnancy in													
		1			2		3			4		5		6	
					Caffeine (mg/kg)		Tricaprylin (control)	Caffeine (mg/kg)		Tricaprylin (control)	Caffeine (175 mg/kg 2)	Water (control)	Caffeine (200 mg/kg)	Control	Caffeine in drinking water [illegible]
					[illegible]	240		168	182						
[illegible]		[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	0·00	0·00	[illegible]	0·06	0·13	0·00	0·25	0·21
[illegible]		[illegible]	[illegible]	[illegible]	0·00	[illegible]	[illegible]	0·00	0·00	[illegible]	0·21	0·23	0·00	0·42	0·34
[illegible]			[illegible]	[illegible]	0·00	[illegible]	[illegible]	0·00	0·00	[illegible]	[illegible]	0·30	0·24	0·39	[illegible]
[illegible]							[illegible]	0·00	[illegible]	[illegible]	[illegible]	0·67	0·14	0·48	[illegible]
[illegible]							0·18	0·23	0·21	[illegible]	[illegible]	0·33	0·17	0·36	[illegible]
[illegible]							[illegible]	0·00	0·00	[illegible]	0·06	0·30	0·50	0·47	[illegible]
[illegible]							0·17	[illegible]	0·00	[illegible]	0·49	0·69	0·46	0·53	[illegible]
[illegible]							[illegible]	[illegible]	0·00	[illegible]	0·22	0·00	0·14	[illegible]	[illegible]
Unweighted overall mean			[illegible]	[illegible]	0·00	0·10	0·10	0·07	0·06	0·08	0·19	0·33	0·21	0·38	0·36
Unweighted standard deviation of means		[illegible]	0·14	0·14	0·00	0·10	0·09	0·10	0·11	0·09	0·14	0·24	0·07	0·14	0·15

of dose *v.* weeks using matrix inversion techniques was performed on each set of data (Table 5). As can be seen, no significant dose-related differences were detected between the total implants in caffeine-treated and control animals in these analyses (Fig. 2). The overall within-experiment variance was 6·04 and with this internal variance, the difference between groups of 20 animals that can be detected as significant at the 5% level must exceed 1·6 total implants. There were no individual differences as large as this nor, in general, was there any systematic variation in the numbers of total implants. As previously reported (Epstein *et al.* 1970a), the distribution of early deaths is asymmetrical and Poisson in type, and it was necessary, therefore, to transform these data prior to analysis of variance. Analysis of dose *v.*

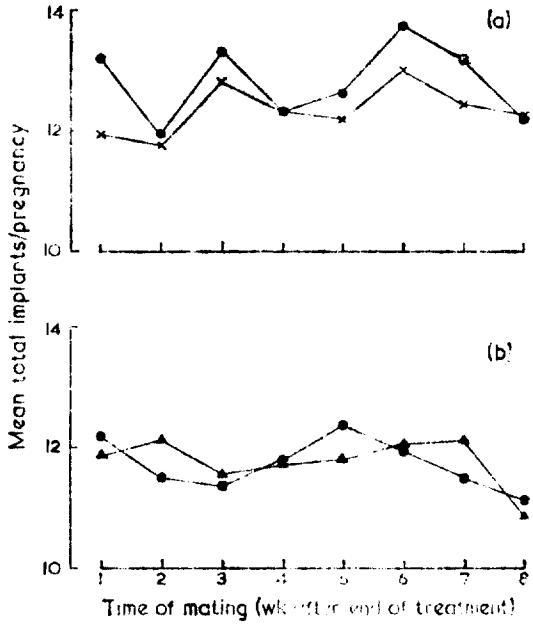

Fig. 2. Effect of caffeine on total implants following (a) [illegible] Total numbers of implants pregnant mouse following treatment of male [illegible] of 175 mg caffeine [illegible] 0·1% caffeine in the [illegible] compared with values for the corresponding controls (●).

weeks (Table 6) did not reveal any significant or consistent difference between the incidence of early deaths in control and caffeine-treated mice in either acute or 8-wk studies. As indicated by the standard deviations (Table 5), a tendency to variability in the number of early foetal deaths was apparent in both control and caffeine-treated groups.

A reduction in the number of total implants below normal control levels, which are considered to be eight or more implants in the strain of mice studied in these laboratories (Epstein *et al.* 1969), indicates the occurrence of pre-implantation losses. In the acute and 8-wk studies, this occurred only sporadically in both caffeine-treated and control mice. There was no indication of any drug- or dose related effect.

Tests on caffeine in combination with known [illegible]

Toxicity. Mortality in the male mice was low and sporadic and showed no relation to dose in either the acute or 8-wk experiments (Table 2).

Table 6. *Analysis of variance for studies of acute and 8-wk caffeine administration*

Source of variation	Total no. of implants/pregnancy				No. of early deaths/pregnancy†			
	Degrees of freedom	Sum of squares	Mean square	F ratio	Degrees of freedom	Sum of squares	Mean square	F ratio
Experiment no. 1								
Total	105	462·08			105	13·07		
Dose (adjusted)	1	0·01	0·01	0·001	1	0·08	0·08	0·77
Weeks (adjusted)	2	6·41	3·20	0·714	2	0·53	0·26	2·30
Interactions	2	6·97	3·48	0·777	2	0·85	0·42	3·68*
Within sub-classes	100	448·61	4·48		100	11·52	0·11	
Experiment no. 2								
Total	132	813·66			132	63·84		
Dose (adjusted)	2	20·68	10·34	1·63	2	0·22	0·11	0·23
Weeks (adjusted)	2	0·26	0·13	0·02	2	1·64	0·82	1·70
Interactions	4	5·30	1·32	0·20	4	2·10	0·52	1·08
Within sub-classes	124	786·46	6·34		124	59·96	0·48	
Experiment no. 3								
Total	338	1455·25			338	52·71		
Dose (adjusted)	2	2·78	1·39	0·36	2	0·18	0·09	0·60
Weeks (adjusted)	7	134·93	19·27	5·04*	7	2·[illegible]	0·35	2·31*
Interactions	14	115·01	8·21	2·14	14	2·31	0·16	1·08
Within sub-classes	315	1203·92	3·82		315	47·[illegible]	0·15	
Experiment no. 4								
Total	342	1969·15			342	108·79		
Dose (adjusted)	1	15·57	15·57	2·71	1	0·89	0·89	2·84
Weeks (adjusted)	7	67·33	9·61	1·67	7	3·02	0·43	1·37
Interactions	7	13·32	1·90	0·33	7	2·46	0·35	1·12
Within sub-classes	327	1872·93	5·72		327	102·49	0·31	
Experiment no. 5								
Total	207	1045·07			207	112·54		
Dose (adjusted)	1	1·51	1·51	0·30	1	0·52	0·51	0·96
Weeks (adjusted)	7	21·36	3·05	0·61	7	7·11	1·01	1·90
Interactions	7	72·89	10·41	2·11*	7	2·83	0·40	0·75
Within sub-classes	192	949·59	4·93		192	102·40	0·53	
Experiment no. 6								
Total	300	1199·11			300	184·41		
Dose (adjusted)	1	0·37	0·37	0·09	1	0·10	0·10	0·16
Weeks (adjusted)	7	49·11	7·01	1·75	7	3·76	0·53	0·85
Interactions	7	8·00	1·14	0·28	7	3·58	0·51	0·82
Within sub-classes	285	1141·64	4·00		285	176·96	0·62	

* Significant at 5% level.

† Data transformed by Freeman–Tukey Poisson transformation before analysis.

Effect on pregnancy rates. In acute experiments, the pregnancy rates for females mated with males treated with caffeine and a mutagen did not generally differ significantly from those mated with males treated with the mutagen alone, except for matings in wk 1 and sometimes in wk 2 (Fig. 3; Table 7). At these times, females mated with males given caffeine and a mutagen had consistently and significantly lower pregnancy rates. Differences at other times were sporadic.

In experiments involving 8-wk administration of caffeine, variation was considerable and was not related to dose or time (Table 8). There was no indication of any consistent reduction in pregnancy rates in the combined caffeine-mutagen experiments compared with the effects of X-irradiation alone (Fig. 3).

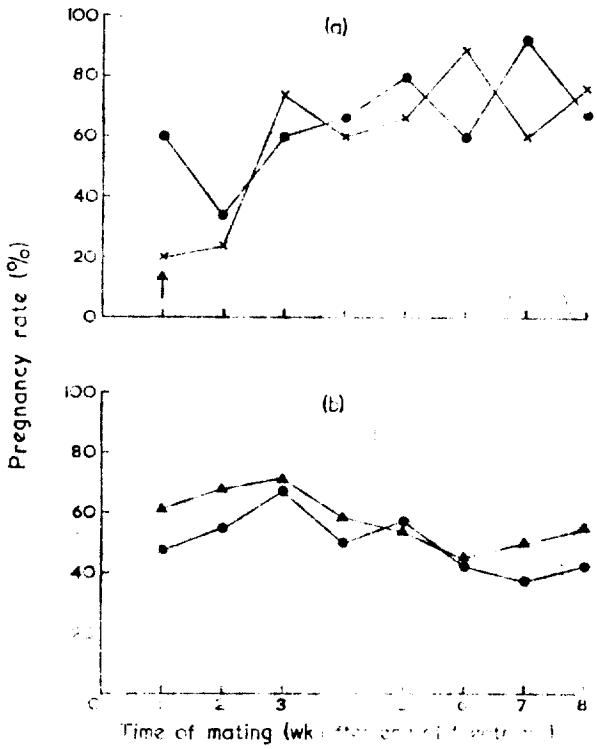

FIG. 3. Effect of caffeine-mutagen interaction on pregnancy rate in mice. (a) Arrows indicate a statistically significant difference ($P < 0.05$) between the group given a single intraperitoneal injection of 1·25 mg TEPA/kg and 200 mg caffeine/kg (×) and controls given the same dose of TEPA alone (●). (b) Values following X-irradiation of males with 200 r, and administration of 0·2% caffeine in the drinking water for 8 wk prior to mating (▲) are compared with controls treated only with X-rays (●).

Mean total implants and early foetal deaths. Total implants, including living foetuses and early foetal deaths, were scored individually for each pregnant mouse and weekly averages were determined for each experimental group (Figs 4 & 5; Tables 9–12). Acute and 8-wk data were analysed for variance of dose *v.* weeks, using matrix inversion techniques (Table 13). Significant variation over weeks was noted in most of the experiments, as was to be expected since all males were treated with a known mutagen. However, no significant differences in these parameters were detected between classes, with the exception of one instance of borderline significance (Table 13, experiment no. 7). Examination of the data and analysis

Table 7. *Percentage pregnancy in acute caffeine-interaction studies*

Time of mating (wk after treatment)	Experiment no.	Incidence of pregnancy (%) in 1			2				3				4		5	
	Treatment	MMS (50 mg/kg) + feed (mg/kg)			TEPA (0·312 mg/kg) + caffeine (mg/kg)		TEPA (1·25 mg/kg) + caffeine (mg/kg)		X-ray (50 r.) + caffeine (mg/kg)		X-ray (200 r.) + caffeine (mg/kg)		X-ray (200 r.) + caffeine (mg/kg)		X-ray (250 r.) + caffeine (mg/kg)	
		0	168	192	0	200	0	200	0	200	0	200	0	168	0	175 × 2
1		70	19	58	47	13	60	20	47	13	47	13	67	41	69	33
2		76	33	42	100	33	33	23	73	40	47	47	59	52	63	53
3		78	67	42	67	70	60	73	73	63	87	43	67	85	50	68
4		74	90	83	89	63	67	60	67	70	80	70	41	81	44	62
5		78	57	75	89	73	80	67	67	70	73	53			51	58
6		67	76	58	33	73	60	90	73	80	67	60			40	35
7		83	86	67	67	73	93	60	67	63	67	70			29	26
8		75	76	75	80	70	67	77	100	57	80	87			56	44

MMS = methyl methanesulphonate

Table 8. *Percentage pregnancy in interaction studies involving 8-wk administration of caffeine*

Time of mating (wk after treatment)	Experiment no.	Incidence of pregnancy (%) in 6				7							
	Treatment	X-ray (200 r.) + caffeine (% in drinking water)				X-ray (200 r.) − caffeine (% in drinking water)				X-ray (50 r.) − caffeine (% in drinking water)			
		0	0·05	0·1	0·2	0	0·1	0·2	0·4	0	0·1	0·2	0·4
1		80	85	77	65	47	48	61	47	56	55	49	44
2		83	83	77	59	54	70	68	33	75	62	54	58
3		77	63	77	67	67	73	72	71	63	75	70	82
4		77	63	70	48	48	62	58	56	77	86	67	80
5		57	37	83	48	57	60	54	60	72	77	59	64
6		57	33	40	41	40	45	44	29	78	81	70	69
7		37	22	57	23	35	48	55	41	65	54	57	64
8		63	59	70	56	40	55	57	48	60	60	54	49

Table 9. *Mean total implants/pregnancy in acute caffeine-interaction studies*

Time of mating (wk after treatment)	Experiment no.	1			2				3				4		5	
	Treatment	MSM (50 mg/kg) ± caffeine (mg/kg)			TEPA (0·312 mg/kg) ± caffeine (mg/kg)		TEPA (1·25 mg/kg) ± caffeine (mg/kg)		X-ray (50 r.) ± caffeine (mg/kg)		X-ray (200 r.) ± caffeine (mg/kg)		X-ray (200 r.) ± caffeine (mg/kg)		X-ray (250 r.) ± caffeine (mg/kg)	
		0	168	192	0	200	0	200	0	200	0	200	0	168	0	175×2
1		9·7	[illegible]	[illegible]	12·7	13·3	11·4	8·7	11·2	13·5	9·6	15·3	10·3	12·5	11·4	11·8
2		9·9	[illegible]	[illegible]	11·2	9·1	8·0	7·6	11·6	12·7	10·1	12·2	10·6	11·4	10·9	10·9
3		11·3	[illegible]	12·2	12·9	11·1	11·3	10·3	11·8	11·7	10·9	11·1	9·8	9·9	10·4	10·4
4		11·5	[illegible]	[illegible]	[illegible]	10·6	10·8	11·8	12·7	10·6	11·1	12·0	10·4	10·0	10·1	9·4
5		11·0	[illegible]	11·1	12·3	11·7	10·9	11·8	12·5	11·2	9·6	11·6			9·3	7·8
6		12·3	12·1	10·9	12·0	11·6	10·0	10·9	11·8	12·0	11·0	10·0			7·6	6·8
7		12·6	11·8	14·4	12·0	11·3	10·6	12·1	11·5	12·0	12·6	10·6			5·8	8·1
8		12·3	12·1	10·6	12·2	13·0	12·4	11·6	12·9	12·0	12·4	12·1			11·0	11·6

MMS = methyl methanesulphonate

Table 10. *Mean total implants/pregnancy in interaction studies involving 8-wk administration of caffeine*

Time of mating (wk after treatment)	Experiment no.	6				7							
	Treatment	X-ray (200 r.) ± caffeine (% in drinking water)				X-ray (200 r.) ± caffeine (% in drinking water)				X-ray (50 r.) ± caffeine (% in drinking water)			
		0	0·05	0·1	0·2	0	0·1	0·2	0·4	0	0·1	0·2	0·4
1		10·7	10·8	11·1	11·4	10·6	11·2	11·5	11·5	12·2	11·1	11·0	11·9
2		11·2	10·7	10·7	11·6	10·0	10·8	10·6	11·9	11·9	10·4	12·4	12·7
3		11·2	10·2	10·7	10·9	9·9	8·3	8·7	8·5	9·9	10·9	11·0	10·8
4		8·7	9·5	9·9	9·2	10·3	9·7	10·7	9·1	12·2	11·6	11·5	11·4
5		11·0	10·2	10·6	10·4	10·0	10·4	10·7	10·1	12·2	11·0	12·1	11·6
6		6·7	[illegible]	7·6	10·2	7·8	8·9	8·7	10·1	11·2	10·5	11·3	11·2
7		9·0	8·7	8·4	10·5	7·4	11·0	10·8	9·9	12·0	12·8	12·9	12·6
8		10·0	11·5	11·0	11·4	11·2	10·9	11·3	11·7	11·4	10·9	10·6	11·9

Table 11. *Mean early foetal deaths/pregnancy in acute caffeine-interaction studies*

Time of mating (wk after treatment)	Experiment no.	1			2				3				4		5	
	Treatment	MMS (5 [illegible]) + caffeine (mg/kg)			TEPA (0·312 mg/kg) + caffeine (mg/kg)		TEPA (0·25 mg/kg) + caffeine (mg/kg)		X-ray (50 r.) + caffeine (mg/kg)		X-ray (200 r.) + caffeine (mg/kg)		X-ray (200 r.) + caffeine (mg/kg)		X-ray (250 r.) + caffeine (mg/kg)	
		0	1·[illegible]	[illegible]	0	200	0	200	0	200	0	200	0	168	0	175 · 2
1		0·74	[illegible]	[illegible]	0·14	0·00	0·00	0·00	0·43	0·25	0·00	0·00	0·61	0·18	0·52	0·15
2		0·26	[illegible]	[illegible]	1·27	1·11	3·60	4·43	0·18	0·17	1·00	0·43	0·56	0·14	0·77	0·28
3		[illegible]	0·89	0·00	1·10	0·76	2·55	3·65	0·45	0·79	2·77	2·07	1·72	1·39	2·04	1·83
4		0·20	0·60	0·00	0·00	0·79	0·50	0·67	0·40	0·71	0·75	1·50	0·82	0·73	0·76	0·51
5		0·00	0·17	0·22	0·75	0·18	0·42	0·15	0·50	0·33	1·36	0·56			1·09	0·60
6		0·17	0·13	0·27	0·40	0·32	0·22	0·56	0·09	0·75	0·50	0·50			0·28	0·65
7		0·05	0·28	0·25	1·10	0·45	0·43	0·50	0·20	0·47	0·50	0·43			0·23	0·13
8		0·78	0·25	0·00	0·17	0·58	0·80	0·09	0·67	0·35	0·42	0·19			0·11	0·12

MMS = methyl methanesulphonate

Table 12. *Mean early foetal deaths/pregnancy in interaction studies involving 8-wk administration of caffeine*

Time of mating (wk after treatment)	Treatment	6				7							
		X-ray (200 r.) + caffeine (% in drinking water)				X-ray (200 r.) + caffeine (% in drinking water)				X-ray (50 r.) + caffeine (% in drinking water)			
		0	0·05	0·1	0·2	0	0·1	0·2	0·4	0	0·1	0·2	0·4
1		0·79	0·83	1·09	1·35	1·60	1·40	1·90	1·60	1·13	0·88	0·57	0·50
2		0·64	1·01	1·83	2·13	1·06	1·19	1·56	1·27	0·84	0·49	0·41	0·85
3		2·00	2·59	2·57	1·22	2·43	1·36	1·85	1·59	0·58	0·37	0·53	0·41
4		1·61	1·82	1·33	1·08	1·90	0·57	1·20	0·32	0·65	0·39	0·61	0·33
5		1·77	2·10	1·76	1·92	3·03	1·40	2·13	1·74	0·56	0·64	0·66	0·38
6		0·82	1·22	0·33	0·82	0·57	0·85	0·50	0·67	0·28	0·78	0·71	0·45
7		0·18	0·17	0·47	0·33	0·38	0·50	0·57	0·18	0·23	0·68	0·48	0·52
8		1·32	0·31	0·33	0·67	0·67	0·36	0·45	0·95	0·28	0·21	0·21	0·55

Table 13. *Analysis of variance for interaction studies involving acute and 8-wk caffeine administration*

Source of variation	Total no. of implants/pregnancy				No. of early deaths/pregnancy†			
	Degrees of freedom	Sum of squares	Mean square	F ratio	Degrees of freedom	Sum of squares	Mean square	F ratio
Experiment no. 1 (MMS 50 mg/kg)								
Total	320	1716·53			320	128·67		
Dose (adjusted)	2	21·60	10·80	2·65	2	0·69	0·34	0·90
Weeks (adjusted)	7	377·26	53·89	13·23**	7	6·12	0·87	2·27*
Dose × week interaction	14	122·55	8·75	2·15	14	7·28	0·52	1·35
Within sub-classes	297	1209·21	4·07		297	114·33	0·38	
Experiment no. 2 (TEPA 0·312 mg/kg)								
Total	223	983·74			223	203·68		
Dose (adjusted)	1	11·89	11·89	2·88	1	0·37	0·37	0·45
Weeks (adjusted)	7	91·19	13·02	3·15**	7	19·40	2·77	3·34**
Dose × week interaction	7	24·77	3·53	0·85	7	10·74	1·53	1·84
Within sub-classes	208	858·02	4·12		208	172·64	0·83	
Experiment no. 2 (TEPA 1·25 mg/kg)								
Total	219	1768·52			219	400·14		
Dose (adjusted)	1	5·16	5·16	0·71	1	0·02	0·02	0·02
Weeks (adjusted)	7	228·02	32·57	4·52**	7	159·90	22·84	20·05**
Dose × week interaction	7	64·51	9·21	1·27	7	7·42	1·06	0·93
Within sub-classes	204	1469·82	7·20		204	232·41	1·13	
Experiment no. 3 (X-ray 50 r.)								
Total	221	1126·19			221	183·75		
Dose (adjusted)	1	2·12	2·12	0·41	1	0·16	0·16	0·19
Weeks (adjusted)	7	21·40	3·05	0·60	7	3·92	0·56	0·66
Dose × week interaction	7	59·75	8·53	1·68	7	5·28	0·75	0·89
Within sub-classes	206	1041·47	5·05		206	174·31	0·84	
Experiment no. 3 (X-ray 200 r.)								
Total	215	1542·62			215	273·17		
Dose (adjusted)	1	14·86	14·86	2·24	1	3·04	3·04	2·93
Weeks (adjusted)	7	53·94	7·70	1·16	7	54·88	7·84	7·54**
Dose × week interaction	7	147·88	21·12	3·19**	7	6·16	0·88	0·84
Within sub-classes	200	1323·40	6·61		200	207·72	1·03	

Experiment no. 4 (X-ray 200 r.)								
Total	132	774·82			132	194·96		
Dose (adjusted)	1	10·57	10·57	1·89	1	22·4	2·40	1·74
Weeks (adjusted)	3	46·83	15·61	2·79	3	20·36	6·78	4·92**
Dose × week interaction	3	25·56	8·52	1·52	3	0·94	0·31	0·22
Within sub-classes	125	698·12	5·58		125	172·34	1·37	
Experiment no. 5 (X-ray 250 r.)								
Total	416	5418·86			416	573·34		
Dose (adjusted)	1	1·09	1·09	0·10	1	0·38	0·38	0·33
Weeks (adjusted)	7	952·77	136·11	12·49**	7	89·97	12·85	11·10**
Dose × week interaction	7	87·55	12·50	1·14	7	18·95	2·70	2·33*
Within sub-classes	401	4369·22	10·89		401	464·18	1·15	
Experiment no. 6 (X-ray 200 r.)								
Total	552	5042·34			552	897·70		
Dose (adjusted)	3	32·05	10·68	1·28	3	2·68	0·89	0·68
Weeks (adjusted)	7	491·38	70·19	8·43**	7	156·25	22·32	17·03**
Dose × week interaction	21	174·54	8·31	0·99	21	56·11	2·67	2·03
Within sub-classes	521	4336·69	8·32		521	682·72	1·31	
Experiment no. 7 (X-ray 200 r.)								
Total	935	8791·76			935	1556·96		
Dose (adjusted)	3	24·70	8·23	0·97	3	33·16	11·05	7·82**
Weeks (adjusted)	7	722·68	103·24	12·22**	7	181·95	25·99	18·40**
Dose × week interaction	21	399·69	19·03	2·25*	21	62·86	2·99	2·11
Within sub-classes	904	7637·07	8·44		904	1277·03	1·41	
Experiment no. 7 (X-ray 50 r.)								
Total	1129	6286·32			1129	963·00		
Dose (adjusted)	3	44·12	14·70	2·84*	3	1·11	0·37	0·44
Weeks (adjusted)	7	324·76	46·39	8·96**	7	20·15	2·87	3·45**
Dose × week interaction	21	231·18	11·00	2·12	21	26·04	1·24	1·48
Within sub-classes	1098	5682·18	5·17		1098	915·49	0·83	

* Significant at 5% level. ** Significant at 1% level.

† Data transformed by Freeman–Tukey Poisson transformation before analysis.

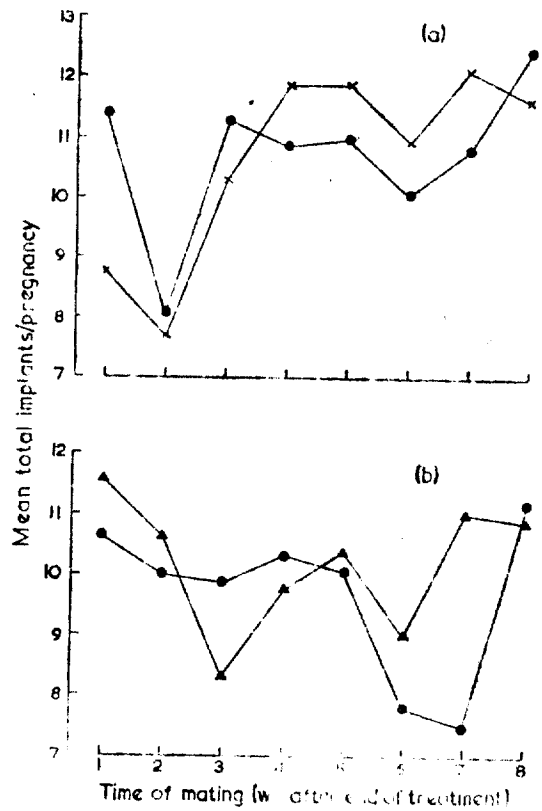

FIG. 4. Effect of caffeine-antiage interaction on total implants in pregnant mice mated [illegible] treated (a) with a single intraperitoneal dose of 1·25 mg TEPA/kg and 200 mg caffeine/kg (×) or 1·25 mg TEPA/kg alone (●) and (b) with X-rays (200 r.) and 0·2% caffeine in the drinking water for 8 wk prior to mating (▲) or X-rays alone (●).

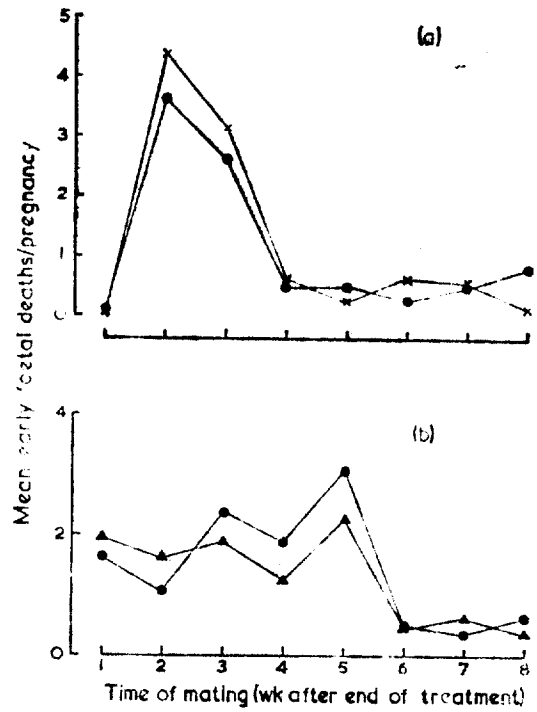

FIG. 5. Effect of caffeine–mutagen interaction on number of early foetal deaths in pregnant mice mated with males treated with (a) a single intraperitoneal dose of 1·25 mg TEPA/kg and 200 mg caffeine/kg (×) or 1·25 mg TEPA/kg alone (●) and (b) X-rays (200 r.) and 0·2% caffeine in the drinking water for 8 wk prior to mating (▲) or X-rays alone (●).

of variance over dose for each week indicated that the variation was not dose-dependent and was not caffeine-induced.

Numbers of early foetal deaths were scored for each pregnant mouse and weekly means were determined (Fig. 5; Tables 11 & 12). As previously reported, the distribution of early deaths is asymmetrical and Poisson in type and data were therefore transformed prior to analysis of variance of dose v. weeks (Table 13). As noted with total implants, significant variation occurred over weeks in most experiments. In only one instance, however, was there any significant difference between doses. Analysis over dose for each week of the early death data for the 200 r. X-ray group of experiment no. 7 revealed that the variation was not dose-dependent and was not caffeine-induced.

A reduction in the total numbers of implants below control levels, which are considered to be eight or more implants, can be taken to indicate pre-implantation losses. For each mutagen tested in these experiments pre-implantation losses occurred most frequently in association with the greatest effects on total numbers of implants and early foetal deaths. In general, there were no significant differences between the incidence of pre-implantation losses in groups receiving caffeine and a mutagen and those receiving a mutagen alone.

DISCUSSION

In these experiments, administration of non-toxic single or repeated doses of caffeine to adult male mice produced no distinct mutagenic effects, as expressed directly by an increase in early foetal deaths or indirectly by pre-implantation losses. Although there was no

significant increase in the number of early deaths in the test groups, the incidence in both control and caffeine-treated groups was somewhat variable. Pre-implantation losses occurred only sporadically and were not related to caffeine administration. No significant or systematic changes were detected in the total number of implants in the test groups. Similarly administration of non-toxic single or repeated doses of caffeine did not synergize the mutagenic effects of known mutagens acutely administered, as measured directly by an increase in early foetal deaths or indirectly by pre-implantation losses.

The effects of caffeine on pregnancies were not well defined. Pregnancies were definitely reduced in females mated to caffeine-treated males, although the effect tended to be inconsistent in relation to dose and time of mating. Following repeated caffeine administration in the combination experiments, no effects greater than those obtained with X-rays alone could be demonstrated. However, in the acute experiments there was a consistent reduction in pregnancy rates following treatment with caffeine in the early weeks of mating, corresponding to the similar trend noted when caffeine was tested alone. It appears that caffeine reduces the pregnancy rate in females mated with males exposed during the post-meiotic stages of spermatogenesis. However, this effect may be obscured by concomitant effects induced by known mutagens. These data confirm previous reports on the failure of caffeine to induce mutagenic effects *in vivo* in mammals. Additionally, under the conditions of test, caffeine failed to induce synergistic mutagenic effects with X-rays or with alkylating agents.

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Inaptitude de la caféine à déclencher des effets mutagenes et à agir en synergie avec les effets de mutagènes connus, chez la souris

Résumé – Administrée à dose aiguë ou pendant 8 semaines à des souris mâles, avant l'accouplement, la caféine n'a pas déterminé d'effet mutagène au cours de l'essai, essentiellement létal comme le prouvent directement les morts prématurées de fœtus et indirectement l'augmentation des pertes de pré-implantations. De plus, ni la dose aiguë ni l'administration pendant 8 semaines n'ont eu d'interaction avec les effets de mutagènes connus, en l'occurrence le méthanesulfonate de méthyle, le TEPA et les rayons X.

Die Unfähigkeit des Koffeins, mutagene Wirkungen einzuleiten oder die Wirkungen bekannter Mutagene in Mäusen synergistisch zu verstärken

Zusammenfassung – Männlichen Mäusen akut oder auf die Dauer von 8 Wochen vor der Paarungszeit verabreichtes Koffein hatte keine mutagene Wirkung bei dem vorherrschend letal verlaufenen Versuch, die direkt durch die Feststellung vermehrten frühzeitigen Absterbens des Fetus oder indirekt durch Feststellung vermehrter Verluste befruchteter Eizellen vor der Implantation erfasst wurde. Ausserdem synergisierte weder die akute noch die 8wöchige Verabreichung von Koffein die Wirkungen der bekannten Mutagene Methylmethansulfonat, TEPA oder Röntgenstrahlen.

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Effects of Coffee Ingestion on Oral Glucose Tolerance Curves in Normal Human Subjects

By LEONARD J. FEINBERG, HERSCHEL SANDBERG, OSCAR DE CASTRO AND SAMUEL BELLET

Oral glucose tolerance tests were performed on 23 normal subjects and then repeated one week later. On one occasion, the test meal consisted of glucose dissolved in water and flavored with lemon juice; on the other occasion, 5 Gm. of instant coffee were also added to the meal. The order of administration of the respective meals was randomized. Serial blood samples were obtained and analyzed for blood glucose concentration, serum free fatty acid levels and the serum immunoreactive insulin values. Paired comparisons of the data were made and the following results were obtained: (1) The subjects ingesting coffee plus glucose had significantly lower blood glucose levels 30 and 60 minutes postprandium than those consuming the glucose solution without coffee. (2) Three hours after ingestion of the test meal, the free fatty acid levels of the subjects receiving coffee with glucose were significantly higher than those receiving glucose without coffee. (3) No statistically significant differences between the two groups were found at any time period for the serum immunoreactive insulin levels. It is possible that coffee ingestion reduced the peak postprandial blood glucose levels by mobilizing a hormone from the gastrointestinal tract such as secretin, pancreozymin, or the newly discovered substance with glucagon-like immunoreactivity described by Unger et al.[37] (Metabolism 17: No. 10, October, 916–922, 1968)

ALTHOUGH THE EFFECTS of methyl xanthines on the kidney and central nervous system have been studied in great detail,[1-3] relatively little is known about the ways in which these drugs influence carbohydrate metabolism.[4] Recent studies from our laboratory[5,6] have shown that subjects who have ingested caffeine show a significant and prolonged rise in serum free fatty acid levels. Since Butcher and Sutherland[7] have shown that methyl xanthines block the inactivation of cyclic 3′,5′-AMP, a ribonucleotide of great

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Table 1.—*Effects of Coffee Ingestion on Oral Glucose Tolerance Curves in Humans*

Minutes After Ingestion of Test Meal	Number of Subjects	Blood Glucose Concentration of Control Subjects (mg. %)	Blood Glucose Concentration of Subjects Ingesting Coffee with Test Meal (mg. %)	Mean Difference	Variance	Standard Error of the Mean	t	P
0	23	80.3 ± 9.0*	79.2 ± 10.9*	1.1				N.S.†
30	18	139.1 ± 25.4	117.7 ± 18.6	21.2	694.30	6.21	3.41	<0.01
60	23	121.7 ± 24.3	98.6 ± 24.0	23.2	615.06	5.17	4.48	<0.001
90	15	90.1 ± 25.7	83.0 ± 12.8	7.1				N.S.
120	23	77.1 ± 21.7	72.6 ± 30.3	4.5				N.S.
180	23	68.2 ± 14.1	72.8 ± 12.3	4.6				N.S.

*Mean ± S.D.

†Differences not statistically significant at the 5 percent level for a paired comparison.

importance in glycogenolysis, we decided to study the effects of coffee on the metabolism of glucose in human subjects.

Materials and Methods

All 23 subjects employed in this study were normal, healthy adults (15 males, and 8 females). The study group consisted predominately of students and technicians, 19–23 years of age; they were placed on a diet containing at least 300 Gm. of carbohydrate 3 days prior to the initial study and were advised to maintain a similar diet when the study was repeated one week later. No food was ingested after 10 p.m. of the night preceding the test. Smoking was prohibited. The following morning 1 Gm. of glucose per kilogram of body weight was administered orally to the test subject. The glucose was dissolved in 400 ml. of water flavored with lemon juice. On 1 of the 2 testing days, 5 Gm. of instant coffee containing 220 mg. of caffeine was administered along with the glucose. The number of subjects receiving coffee with glucose as the first test meal was equal to the number receiving glucose without coffee initially and the order of administration of the respective meals was randomized.

Blood samples were obtained prior to the administration of the test meal and at serial intervals thereafter. In the earlier studies, these were obtained at hourly intervals. Later, test samples were also taken 30 and 90 minutes postprandially as well. Blood glucose concentrations were determined by the method of Somogyi-Nelson.[8] Serum free fatty acids were determined in the initial 5 subjects by a modification of the method of Gordon[9] and in the latter 18 subjects by the method of Antonis adapted for the autoanalyzer.[10] The serum immunoreactive insulin (IRI) was assayed by the method of Hales and Randle,[11] a radioimmunoassay utilizing the principle of competitive inhibition and 2 antibodies, one to form a soluble complex with radioactive (and non-radioactive) insulin; the other to precipitate this complex and thus separate it from free radioactive insulin.

Results

Effects of Coffee Ingestion on Oral Glucose Tolerance Curve

From Table 1, it can be seen that the glucose tolerance curve was significantly altered when 5 Gm. of coffee were added to the test meal. The zero time samples showed no important differences between the control and the coffee-treated group. Thirty minutes after ingestion of the test meal, the subjects receiving glucose without coffee averaged 139.1 ± 25.4 mg. per

Table 2.—*Effects of Coffee Ingestion on Serum Free Fatty Acid Levels During Course of Glucose Tolerance Test*

Minutes after Ingestion of Test Meal	Number of Subjects	FFA Concentration of Control Subjects (% of zero time sample)	FFA Concentration of Coffee Ingesting Subjects (% of zero time sample)	Mean Difference	Variance	Standard Error of the Mean	t	P
30	13	84.5 ± 18.0*	82.8 ± 18.5*	−1.7				N.S.†
60	18	75.7 ± 24.3	81.1 ± 24.8	5.4				N.S.
90	11	67.8 ± 20.3	67.9 ± 15.4	0.1				N.S.
120	18	73.3 ± 27.5	82.7 ± 23.5	9.4				N.S.
180	15	86.5 ± 33.3	117.6 ± 36.8	20.9	1281	9.565	2.196	<0.05

*Mean ± S.D.

†Differences not statistically significant at the 5 percent level for a paired comparison.

cent while those also receiving 5 Gm. of instant coffee averaged 117.7 ± 18.6 mg. per cent. One hour after the test meal, the control group averaged 121.7 ± 24.5 mg. per cent, while the coffee-treated group averaged 98.6 ± 24.0 mg. per cent. T tests applied to a paired comparison gave values of 3.41 and 4.81, respectively, with P values of <0.01 and <0.001. At 90, 120 and 180 minutes after administration of the test meal, no significant differences between the two groups were found.

Effects of Coffee Ingestion on Serum Free Fatty Acid Levels During Oral Glucose Tolerance Test

Table 2 shows the effect of adding coffee to the test meal on serum free fatty acid concentrations of human subjects during an oral glucose tolerance test. Both groups showed sharp drops in the free fatty acid levels after ingesting the test meal, but no significant differences in the free fatty acid concentrations of the two groups were found until 3 hours postprandium. At this time, the control group averaged 86.5 ± 33.3 per cent of the zero time values, while the group receiving coffee averaged 117.3 ± 36.8 per cent. T test applied to a paired comparison gave a value of 2.496 with a P value of <0.05.

Effect of Coffee Ingestion on Immunoreactive Insulin Levels of Serum During Oral Glucose Tolerance Test

From Table 3, it can be seen that peak immunoreactive insulin values were found in both groups at 30 and 60 minutes postprandium and fell rapidly thereafter. No statistically significant differences in the immunoreactive insulin values between the two groups were found during the 3 hours of the glucose tolerance test.

Discussion

From the results shown above, the addition of 5 Gm. of instant coffee to glucose tolerance test meal produces a significant effect on both the free fatty acid concentration of the serum and the blood glucose concentration. The

Table 3.—*Effects of Coffee Ingestion on Serum Immunoreactive Insulin Levels During Course of Glucose Tolerance Test*

Minutes after Ingestion of Test Meal	Number of Subjects	Immunoreactive Insulin Levels of Control Subjects (μU/ml.)	Immunoreactive Insulin Levels of Subjects Ingesting Coffee with Test Meal (μU/ml.)	Mean Difference	P
0	23	15.3 ± 11.2*	13.2 ± 13.4*	2.1	N.S.†
30	17	83.2 ± 56.0	95.6 ± 45.9	12.4	N.S.
60	23	93.9 ± 60.1	85.5 ± 45.1	8.4	N.S.
90	17	64.5 ± 40.8	63.0 ± 42.8	1.5	N.S.
120	22	33.9 ± 24.3	28.1 ± 24.0	5.8	N.S.
180	22	13.0 ± 14.5	13.8 ± 18.4	0.8	N.S.

*Mean ± S.D.

†Differences are not statistically significant at the percent level for a paired comparison.

effect on free fatty acids could possibly have been anticipated since carbohydrate ingestion has been shown to lower serum free fatty acid levels,[9] while caffeine has been found to elevate them.[5,6] When the glucose effect is dissipated and the effect of caffeine persists, the result would be a rise in the free fatty acid levels shown by the 3-hour sample of Table 2.

The effect of coffee ingestion on the glucose tolerance curve, however, was unexpected since several earlier studies seem to suggest a hyperglycemic action of caffeine. Northrup and Parks[12] reported that methylxanthines potentiated the hyperglycemia induced in rats with cyclic 3′,5′-AMP. Kuftinec and Mayer[4] observed that coffee produced a marked elevation of blood glucose in obese hyperglycemic mice. Bömer,[13] Hirayama,[14] and Karger[15] have noted that caffeine produced hyperglycemia and glycosuria in dogs. In a recent study,[16] a group of dental students, receiving caffeine in capsule form, showed significantly higher glucose levels than a control group matched by age and weight. Jankelson et al.[17] have reported that the ingestion of coffee by patients with maturity-onset diabetes results in higher blood glucose levels during an intravenous glucose tolerance test than those found when water was substituted for coffee.

In vitro studies would also lead us to expect a hyperglycemic action of caffeine. Vaughan,[18] as well as Anderson et al.,[19] have shown that caffeine inhibits the uptake of glucose by rat adipose tissue. The latter group[20] has observed that caffeine inhibits the production of $C^{14}O_2$ and the C^{14} lipid incorporation from glucose 1-C^{14}. Sutherland and his associates[21-23] have noted that caffeine increases liver phosphorylase activity and glycogenolysis by inhibiting a phosphodiesterase which destroys cyclic 3′,5′-AMP.

Other studies, however, lend support to our findings. Iancu et al.[24] observed that caffeine, administered subcutaneously, produced a decrease in the fasting blood glucose levels of dystrophic sucklings with total cortical inhibition. Although Kuftinec and Mayer[4] observed that caffeine accentuates the hyperglycemia of obese mice, their lean litter mates were rendered hy-

poglycemic by the drug. Hankiewicz[25] in a study of 226 patients (75 per cent of whom were diabetic) found that the administration of caffeine resulted in a drop in the fasting blood sugar levels. Deakins,[26] who performed 8 glucose tolerance curves in a single subject, reported that large doses of caffeine depress the peak of the human sugar-tolerance curve.

The mechanisms by which coffee lowered the peak values of the postprandial blood sugar are unknown. Several recent studies, however, may provide clues for this action. Lambert et al.[27] speculated that insulin release might be dependent on the intracellular accumulation of cyclic 3′,5′-AMP. When they added caffeine to an incubation medium containing foetal pancreatic tissue in the absence of glucose, they observed a sharp increase of radioimmunoassayable insulin released into the medium. When caffeine was added along with glucagon or tolbutamide, the insulin released from the pancreatic tissue was greatly increased. Perhaps caffeine works similarly in vivo, blocking the action of phosphodiesterase, prolonging the action of the cyclic nucleotide, thus stimulating an increase in insulin released from the pancreas resulting in a subsequent hypoglycemic action. Such a mechanism would offer an explanation for the significant decrease in blood glucose found in adrenalectomized rats treated with dihydroergotamine and subsequently with 3′,5′-AMP.[12] The lack of a significant increase in the peripheral blood insulin levels in our subjects would not necessarily negate this hypothesis, since Anderson et al.[28] have clarified mechanisms by which increasing insulin concentrations in the pancreatic veins may be associated with inhibition, by the liver, of insulin output into the periphery.

Other mechanisms, however, may be operative. Since coffee contains noncaffeine compounds such as volatile aromatic, oils, furfuryl alcohol and tannin-like substances which have been stated to be local gastric irritants,[29] it is possible that these agents produced a state of hypermotility, resulting in a rapid transit time of glucose through the gut and hence defective absorption of the test meal. Jankelson,[17] however, failed to find evidence for such gastric irritation. Moreover, studies recently completed in our laboratory have shown that similar lowering of the postprandial blood sugar can be produced in dogs by adding a solution of caffeine sodium benzoate alone to the test meal.

Several groups[30-33] have shown that insulin-like activity for a given glucose load is considerably higher when glucose is administered orally rather than intravenously. Perley and Kipnis,[33] in a well-planned study, also showed this difference to exist in patients with portacaval shunts. They therefore postulated that some mechanism, located in the upper gastrointestinal tract, mediated in part, the insulin response to an oral glucose load. Glucagon[34-36] and pancreozymin[36] have been found to stimulate the secretion of insulin. Unger et al.[37] recently extracted from the jejunum a substance with glucagon-like immunoreactivity but devoid of hyperglycemic, glycogenolytic or adenylcyclase activating activity, which is capable of stimulating insulin release. Gley and Hazard[38] injected dilute hydrochloric acid into the duodenum of dogs and found that blood from these animals reduced the blood sugar of recipient dogs. Zunz and Le Barre[39] performed pancreatico-jugular anast-

amoses on adrenal decapsulated dogs and found that secretin, injected intravenously into the donor animal, produced hypoglycemia in the recipient dog. Shay and Gershon-Cohen[40] found that stimulation of the duodenal mucosa with hydrochloric acid, fat, and hypertonic salt solutions could block, or significantly reduce, alimentary hyperglycemia. Since caffeine is known to profoundly augment gastric secretion in man, with peak levels of free acid appearing 40–50 minutes after administration of caffeine[41] it is possible that this mechanism might play an important role in the reduced post alimentary blood sugar peaks found in our study of normal subjects.

The results of the present study are of interest. They show that marked decreases in blood glucose concentration may occur following the ingestion of coffee without concomitant changes in the serum immunoreactive insulin levels of the peripheral blood; that glucose ingestion inhibits, for several hours, caffeine's ability to elevate serum free fatty acids.

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FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date January 8, 1973

Laboratory No. 0901 y
Contract No. FDA 71-260

Sample: Fine white powdered material

Marking: FDA 71-44 (Caffeine)

Examination Requested: Teratologic evaluation of FDA 71-44 in mice.

Procedure: See Appendix I

Results: See Tables 1 through 4 and Appendix II

Conclusion: Subject to reexamination in the light of additional data in progress, the following is concluded:

"The administration of up to 36 mg/kg (body weight) of the test material to pregnant mice for 10 consecutive days had no clearly discernible effect on nidation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

Comment: Attention is called to the fact that this is the eighteenth of a series of reports which will be issued in accordance with the terms of the contract cited above. Eventually, a total of at least 42 compounds will have been tested in 21 pairs; each pair being run concurrently against one sham-treated control and one positive control group. Because of the inherent variability of biological data of the type dealt with here, the accumulation and pooling of sequential sets of control values will greatly enhance the statistical value of the findings and the ultimate reliability of the test results.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgareidge

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Vice President

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups: 171 & 172; 177 through 180

Material: FDA 71-44

Date: December 8, 1972

Laboratory No.: 0901 y

Table 1

Fate Summary

(Mice)

Group	Material	Dose** mg/kg	Total		Surviving at Term	
			Mated	Pregnant	Total	Pregnant[1]
171	Sham	0.0	26	21	26	21
172	Aspirin*	150.0	27	21	27	21
177	FDA 71-44	0.36	26	21	26	21
178	FDA 71-44	1.7	25	20	25	20
179	FDA 71-44	8.0	25	21	25	21
180	FDA 71-44	36.0	25	21	25	21

* Positive Control: 150.0 mg/kg

** Administered as a water solution;

1) Includes all dams examined at term

(Sham group dosed with anhydrous corn oil)

FOOD AND DRUG RESEA LABORATORIES, INC.

Group : 171 & 172; 177 through 180

Material : FDA 71-44

Table 2
Reproduction Data
(Mice)

Date : December 8, 1972

Laboratory No. : 0901 y

Group :	171	172	177	178	179	180
Dose (mg/kg) :	Sham	Aspirin**	0.36	1.7	8.0	36.0
Pregnancies						
Total No.	21	21	21	20	21	21
Died or Aborted (before Day 17)	0	0	0	0	0	0
To term (on Day 17)	21	21	21	20	21	21
Corpora Lutea						
Total No.	333	349	357	321	337	358
Average/dam mated	12.8	12.9	13.4	12.4	13.5	14.3
Live Litters						
Total No.*	21	21	21	20	20	21
Implant Sites						
Total No.	236	233	241	228	238	266
Average/dam*	11.2	11.1	11.5	11.4	11.3	12.7
Resorptions						
Total No.*	11	11	4	14	25	16
Dams with 1 or more sites resorbed	8	8	3	7	11	13
Dams with all sites resorbed	--	--	--	--	1	--
Per cent partial resorptions	38.1	38.1	14.3	35.0	52.4	61.9
Per cent complete resorptions	--	--	--	--	4.76	--
Live Fetuses						
Total No.	218	219	231	210	207	245
Average/dam*	10.4	10.4	11.0	10.5	9.86	11.7
Sex ratio (M/F)	0.80	0.67	0.58	0.65	0.72	0.70
Dead Fetuses						
Total No.*	7	3	6	4	6	5
Dams with 1 or more dead	4	3	4	3	3	5
Dams with all dead	--	--	--	--	--	--
Per cent partial dead	19.1	14.3	19.1	15.0	14.3	23.8
Per cent all dead	--	--	--	--	--	--
Average Fetus Weight, g	0.90	0.84	0.87	0.88	0.83	0.80

* Includes only those dams examined at term.

** Positive Control : 150.0 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 171 & 172; 177 through 180 Laboratory No. 0901 y

Table 3

Material FDA 71-44 Date December 8, 1972

Summary of Skeletal Findings*

(Mice)

Findings / Group No.:	171	172	177	178	179	180
Dose (mg/kg):	Sham	Aspirin**	0.36	1.7	8.0	36.0
Live Fetuses Examined (at term)	151/21	140/19[a]	161/21	147/20	145/20	172/21
Sternebrae						
Incomplete oss.	43/15	48/16	69/17	57/17	39/15	73/20
Scrambled						
Bipartite	9/6	16/10	7/6	3/3	2/2	5/2
Fused						
Extra	1/1			2/2		5/2
Missing	29/12	24/12	19/8	33/9	21/8	53/14
Other						
Ribs						
Incomplete oss.						
Fused/split						
Wavy						
Less than 12	1/1	1/1				1/1
More than 13	26/9	15/7	24/10	15/5	15/5	5/4
Other						
Vertebrae						
Incomplete oss.	3/3	11/8	3/2	2/1	6/3	5/3
Scrambled						
Fused						
Extra ctrs. oss.						
Scoliosis						
Tail defects						
Other		19/9				
Skull						
Incomplete closure			1/1	2/2	3/2	1/1
Missing				1/1		
Craniostosis						
Other						
Extremities						
Incomplete oss.	21/9	19/10	15/8	28/10	30/9	26/8
Missing	6/5	4/4				
Extra						
Miscellaneous						
Hyoid; missing	55/17	37/15	45/15	61/16	37/14	49/19
Hyoid; reduced	12/7	15/7	17/11	15/11	20/13	38/18
Hyoid; bipartite						1/1

* Numerator=Number of fetuses affected; Denominator=Number of litters affected.

** Positive control: 150.0 mg/kg

a) One litter missing

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 171 & 172; 177 through 180 Date December 8, 1972

Material FDA 71-44 Laboratory No. 0901 y

Table 3-a

Summary of Soft Tissue Abnormalities

(Mice)

Group	Material	Dose Level mg/kg	Dam	Number of Pups	Description
			None Observed		

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 171 & 172; 177 through 180

Date December 8, 1972

Table 4

Laboratory No. 0901 y

Species Mice

Average Body Weights *

Group	Material	Dose Level	Day 0	6	11	15	17**
		mg/kg	g				
171	Sham	0.0	31.1	32.0	34.6	43.5	49.0 (21)
172	Aspirin***	150.0	30.1	32.1	33.7	42.2	47.7 (21)
177	FDA 71-44	0.36	31.3	33.5	37.1	44.4	51.0 (21)
178	FDA 71-44	1.7	30.9	33.1	35.9	44.3	50.0 (20)
179	FDA 71-44	8.0	29.2	31.6	33.8	41.0	45.8 (21)
180	FDA 71-44	36.0	31.6	33.1	33.9	42.3	47.2 (21)

* Of pregnant dams
** Number of surviving dams in parentheses (c.f. Table 1)
*** Positive control: 150.0 mg/kg

Appendix I

Teratology Study in Mice

Virgin adult female albino CD-1 outbred mice were individually housed in disposable plastic cages in temperature and humidity-controlled quarters with free access to food and fresh tap water. They were mated with young adult males, and observation of the vaginal sperm plug was considered Day 0 of gestation. Beginning on Day 6 and continuing daily through Day 15 of gestation, the females were dosed with the indicated dosages by oral intubation; the controls were sham treated.

Body weights were recorded on Days 0, 6, 11, 15, and 17 of gestation. All animals were observed daily for appearance and behavior with particular attention to food consumption and weight, in order to rule out any abnormalities which may have occurred as a result of anorexic effects in the pregnant female animal.

On Day 17 all dams were subjected to Caesarean section under surgical anesthesia, and the numbers of implantation sites, resorption sites, and live and dead fetuses were recorded. The body weights of the live pups were also recorded. The urogenital tract of each dam was examined in detail for anatomical normality.

All fetuses were examined grossly for the presence of external congenital abnormalities. One-third of the fetuses of each litter underwent detailed visceral examinations employing 10X magnification. The remaining two-thirds were cleared in potassium hydroxide (KOH), stained with alizarin red S dye and examined for skeletal defects.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Appendix II

Reproduction Data in Mice (Individual)

Group 171

Material Sham

Dose 0.0 mg/kg

Date December 8, 1972

Laboratory No. 0901

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
S 1511	P	17	12	12		6	6		1.06	
S 1512	P	13	12	12		6	6		0.89	
S 1513	NP	0	0						----	
S 1514	P	12	4	4		2	2		0.90	
S 1515	P	13	12	9	2	3	6	1	0.90	
S 1516	P	13	9	9		5	4		0.83	
S 1517	P	14	12	10	2	4	6		0.85	
S 1518	NP	11	0						----	
S 1519	P	18	16	15	1	8	7		0.83	
S 1520	P	18	14	14		11	3		0.89	
S 1521	P	22	10	10		3	7		0.92	
S 1522	P	15	15	12	2	5	7	1	0.85	
S 1523	P	16	11	11		4	7		0.84	
S 1524	P	11	5	5		3	2		0.97	
S 1525	P	7	6	2		1	1	4	1.08	
S 1526	P	14	13	12		6	6	1	1.15	
S 1527	P	13	14	13		6	7	1	0.78	
S 1528	P	13	12	11		3	8	1	0.90	
S 1529	P	14	12	12		3	9		0.74	
S 1530	NP	0	0						----	
S 1531	NP	12	0						----	
S 1532	NP	14	0						----	
S 1533	P	10	10	10		3	7		0.88	
S 1534	P	14	13	12		2	10	1	0.89	
S 1535	P	16	13	12		7	5	1	0.82	
S 1536										Not Assigned
S 1537	P	13	11	11		6	5		0.85	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 172

Material Aspirin

Dose 150.0 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date December 8, 1972

Laboratory No. 0901

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
A 1511	P	17	12	12		6	6		0.79	
A 1512	NP	5	0						----	
A 1513	P	13	10	9	1	5	4		0.78	
A 1514	NP	8	0						----	
A 1515	P	13	9	9		6	3		0.75	
A 1516	P	16	10	8		3	5	2	0.89	
A 1517	P	11	5	5		2	3		0.80	
A 1518	P	14	12	10	1	5	5	1	0.80	
A 1519	P	13	5	3		1	2	2	0.64	
A 1520	P	13	12	12		6	6		0.88	
A 1521	P	13	14	13		6	7	1	0.81	
A 1522	P	15	12	12		5	7		0.85	
A 1523	P	13	10	10		5	5		0.82	
A 1524	P	13	14	13		4	9	1	0.87	
A 1525	NP	8	0						----	
A 1526	P	14	12	12		4	8		0.79	
A 1527	P	14	14	14		4	10		0.78	
A 1528	P	15	12	10	1	5	5	1	1.12	
A 1529	NP	10	0						----	
A 1530	P	11	10	9		4	5	1	1.05	
A 1531	NP	20	0						----	
A 1532	P	13	11	11		3	8		0.89	
A 1533	P	12	12	10		3	7	2	0.82	
A 1534	P	18	15	15		8	7		0.78	
A 1535										Not Assigned
A 1536	NP	14	0						----	
A 1537	P	13	12	12		2	10		0.89	
A 1538	P	10	10	10		1	9		0.76	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 177

Material FDA 71-44

Dose 0.36 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date December 8, 1972

Laboratory No. 0901 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 1001	P	14	14	14		5	9		0.75	
Y 1002	P	12	12	12		7	5		1.08	
Y 1003	P	16	12	12		5	7		0.83	
Y 1004	P	13	10	10		6	4		0.81	
Y 1005	P	12	7	7		3	4		1.21	
Y 1006	P	17	13	13		3	10		0.83	
Y 1007	NP	10	0						----	
Y 1008	P	12	4	4		3	1		0.90	
Y 1009	P	9	10	7	3	3	4		0.99	
Y 1010	P	14	14	14		7	7		0.78	
Y 1011	P	11	11	11		4	7		0.82	
Y 1012	P	18	15	15		8	7		0.85	
Y 1013	P	16	13	12		6	6	1	0.81	
Y 1014	P	12	10	9	1	1	8		0.67	
Y 1015	NP	19	0						----	
Y 1016	P	14	14	13		1	12	1	0.76	
Y 1017	NP	15	0						----	
Y 1018	NP	7	0						----	
Y 1019	NP	9	0						----	
Y 1020	P	20	16	16		5	11		0.76	
Y 1021	P	14	10	10		1	9		0.94	
Y 1022	P	16	15	14	1	1	13		0.71	
Y 1023	P	8	3	3		2	1		0.99	
Y 1024	P	14	9	9		4	5		0.99	
Y 1025	P	17	15	13		5	8	2	0.82	
Y 1026	P	18	14	13	1	7	6		0.87	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 178

Material FDA 71-44

Dose 1.7 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date December 8, 1972

Laboratory No. 0901 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 1031	P	13	12	12		6	6		0.94	
Y 1032	P	15	13	13		4	9		0.69	
Y 1033	P	13	10	10		5	5		0.94	
Y 1034	P	16	12	11	1	6	5		1.10	
Y 1035	P	14	10	10		6	4		0.82	
Y 1036	NP	9	0						----	
Y 1037	P	15	13	13		7	6		0.82	
Y 1038	P	17	14	10		4	6	4	1.11	
Y 1039	P	11	10	9	1	3	6		0.93	
Y 1040	P	12	8	8		3	5		0.76	
Y 1041	NP	0	0						----	
Y 1042	P	12	12	11		4	7	1	0.59	
Y 1043	P	11	8	7		2	5	1	0.98	
Y 1044	P	15	11	11		2	9		0.85	
Y 1045	P	15	12	11		4	7	1	0.95	
Y 1046	P	13	13	9		3	6	4	1.00	
Y 1047	P	16	13	11		6	5	2	0.88	
Y 1048	NP	0	0						----	
Y 1049	NP	12	0						----	
Y 1050	P	13	10	10		2	8		0.74	
Y 1051	NP	16	0						----	
Y 1052	P	14	12	12		2	10		0.88	
Y 1053	P	19	13	12		6	6	1	0.92	
Y 1054	P	19	14	14		6	8		0.94	
Y 1055	P	11	8	6	2	2	4		0.66	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 179

Material FDA 71-44

Dose 8.0 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date December 8, 1972

Laboratory No. 0901 y

Dam No.	Fate*	Corpora Lutea	Implant Sites.	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 1061	P	16	11	11		6	5		0.89	
Y 1062	P	17	10	10		6	4		0.75	
Y 1063	P	17	15	14	1	6	8		0.82	
Y 1064	P	13	11	9		5	4	2	0.89	
Y 1065	P	13	14	12		6	6	2	0.88	
Y 1066	P	17	14	13	1	7	6		0.86	
Y 1067	P	19	9	8		4	4	1	0.81	
Y 1068	P	16	5	5		3	2		0.81	
Y 1069	P	15	15	15		6	9		0.92	
Y 1070	P	10	9	8		1	7	1	0.73	
Y 1071	NP	5	0						----	
Y 1072	P	10	9					9	----	
Y 1073	P	8	10	8		2	6	2	0.81	
Y 1074	P	12	11	6	4	2	4	1	0.46	
Y 1075	NP	14	0						----	
Y 1076	P	11	11	8		3	5	3	1.09	
Y 1077	P	17	13	11		2	9	2	0.63	
Y 1078	P	13	11	10		6	4	1	0.97	
Y 1079	NP	10	0						----	
Y 1080	P	13	10	10		0	10		0.83	
Y 1081	NP	11	0						----	
Y 1082	P	12	11	11		0	11		0.92	
Y 1083	P	15	12	12		6	6		0.89	
Y 1084	P	13	10	9		5	4	1	0.82	
Y 1085	P	20	17	17		6	11		0.77	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 180

Material FDA 71-44

Dose 36.0 mg/kg

Appendix II

Reproduction Data in Mice (Individual)

Date December 8, 1972

Laboratory No. 0901 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 1091	P	15	12	11		5	6	1	0.83	
Y 1092	P	18	13	12		5	7	1	0.74	
Y 1093	P	22	14	14		7	7		0.74	
Y 1094	P	14	15	14	1	6	8		0.83	
Y 1095	P	16	9	6		3	3	3	0.81	
Y 1096	P	17	16	14	1	6	8	1	0.95	
Y 1097	P	18	13	13		8	5		0.90	
Y 1098	P	14	14	12	1	4	8	1	0.89	
Y 1099	P	12	10	9		4	5	1	0.82	
Y 1100	P	16	14	14		7	7		0.87	
Y 1101	P	16	14	13		5	8	1	0.67	
Y 1102	P	16	15	14		6	8	1	0.54	
Y 1103	NP	15	0						----	
Y 1104	P	14	13	13		4	9		0.74	
Y 1105	P	12	12	11		2	9	1	0.77	
Y 1106	P	11	11	10		3	7	1	0.75	
Y 1107	P	13	11	11		3	8		0.56	
Y 1108	P	14	12	10	1	4	6	1	0.78	
Y 1109	NP	14	0						----	
Y 1110	P	13	11	10		5	5	1	0.97	
Y 1111	NP	16	0						----	
Y 1112	NP	0	0						----	
Y 1113	P	10	12	9	1	--	--	2	0.80	
Y 1114	P	13	10	10		4	6		0.95	
Y 1115	P	19	15	15		6	9		0.93	

* P = Pregnant; NP = Not Pregnant

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FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date January 8, 1973

Laboratory No. 0902 y
Contract No. FDA 71-260

Sample: Fine white powdered material

Marking: FDA 71-44 (Caffeine)

Examination Requested: Teratologic evaluation of FDA 71-44 in rats

Procedure: See Appendix I

Results: See Tables 1 through 4 and Appendix II

Conclusion: Subject to reexamination in the light of later findings, the following is concluded:

"The administration of up to 22.5 mg/kg (body weight) of the test material to pregnant rats for 10 consecutive days had no clearly discernible effect on nidation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

Comment: Attention is called to the fact that this is the eighteenth of a series of reports which will be issued in accordance with the terms of the contract cited above. Eventually, a total of at least 42 compounds will have been tested in 21 pairs; each pair being run concurrently against one sham-treated control and one positive control group. Because of the inherent variability of biological data of the type dealt with here, the accumulation and pooling of sequential sets of control values will greatly enhance the statistical value of the findings and the ultimate reliability of the test results.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgareidge

Kenneth Morgareidge, Ph.D.
Vice President

This report is submitted for the exclusive use of the person, partnership, or corporation to whom it is addressed, and neither the report nor the name of these Laboratories nor of any members of its staff, may be used in connection with the advertising or sale of any product or process without written authorization.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups: 171 & 172; 177 through 180

Material: FDA 71-44

Table 1

Fate Summary
(Rats)

Date: December 8, 1972

Laboratory No.: 0902 y

Group	Material	Dose ** mg/kg	Total		Surviving at Term	
			Mated	Pregnant	Total	Pregnant[1]
171	Sham	0.0	24	23	24	23
172	Aspirin*	250.0	24	20	24	20
177	FDA 71-44	0.23	24	24	24	24
178	FDA 71-44	0.68	24	19	24	19
179	FDA 71-44	3.2	24	22	24	22
180	FDA 71-44	22.5	28	23	28	23

* Positive Control: 250.0 mg/kg

** Administered as a water solution;

1) Includes all dams examined at term

(Sham group dosed with anhydrous corn oil)

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group : 171 & 172; 177 through 180

Material : FDA 71-44

Table 2
Reproduction Data
(Rats)

Date : December 8, 1972

Laboratory No. : 0902 y

Group :	171	172	177	178	179	180
Dose (mg/kg) :	Sham	Aspirin**	0.23	0.68	3.2	22.5
Pregnancies						
Total No.	23	20	24	19	22	23
Died or Aborted (before Day 20)	0	0	0	0	0	0
To term (on Day 20)	23	20	24	19	22	23
Corpora Lutea						
Total No.	259	244	266	235	276	311
Average/dam mated	10.8	10.2	11.1	9.79	11.5	11.1
Live Litters						
Total No.*	23	19	24	19	22	23
Implant Sites						
Total No.	235	209	255	216	250	243
Average/dam*	10.2	10.5	10.6	11.4	11.4	10.6
Resorptions						
Total No.*	4	24	3	4	6	5
Dams with 1 or more sites resorbed	2	5	2	3	4	5
Dams with all sites resorbed	--	1	--	--	--	--
Per cent partial resorptions	8.70	25.0	8.33	15.8	18.2	21.7
Per cent complete resorptions	--	5.00	--	--	--	--
Live Fetuses						
Total No.	230	185	252	212	244	237
Average/dam*	10.0	9.25	10.5	11.2	11.1	10.3
Sex ratio (M/F)	0.98	0.80	0.98	0.77	0.85	0.88
Dead Fetuses						
Total No.*	1	--	--	--	--	1
Dams with 1 or more dead	1	--	--	--	--	1
Dams with all dead	--	--	--	--	--	--
Per cent partial dead	4.35	--	--	--	--	4.35
Per cent all dead	--	--	--	--	--	--
Average Fetus Weight, g	3.84	2.76	3.71	3.57	3.86	3.94

* Includes only those dams examined at term.

** Positive Control : 250.0 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 171 & 172; 177 through 180 Laboratory No. 0902 y

Table 3

Material FDA 71-44 Date December 8, 1972

Summary of Skeletal Findings*

(Rats)

Findings / Group No.:	171	172	177	178	179	180
Dose (mg/kg):	Sham	Aspirin**	0.23	0.68	3.2	22.5
Live Fetuses Examined (at term)	162/23	127/19	179/24	149/19	170/22	166/23
Sternebrae						
Incomplete oss.	33/14	83/19	48/17	53/16	21/10	37/14
Scrambled						
Bipartite		6/4		3/2		
Fused						
Extra						
Missing	17/9	104/17	22/8	26/7	11/8	7/4
Other						
Ribs						
Incomplete oss.		2/2				
Fused/split		1/1				
Wavy	1/1	40/12	17/10	8/4	13/9	5/5
Less than 12		2/1	1/1			
More than 13	1/1	91/18		1/1	1/1	2/2
Other						
Vertebrae						
Incomplete oss.	2/2	90/17	27/11	47/14	20/9	10/8
Scrambled		1/1				
Fused						
Extra ctrs. oss.						
Scoliosis						
Tail defects						
Other						
Skull						
Incomplete closure	17/10	63/17	2/2	12/9	4/4	15/6
Missing		5/2				
Craniostosis						
Other			1/1		1/1	
Extremities						
Incomplete oss.		4/3	2/1	2/1		
Missing		2/1	2/2	4/3	2/2	
Extra						
Miscellaneous						
Hyoid; missing	19/10	68/17	30/18	25/12	19/9	26/11
Hyoid; reduced	28/13	17/11	24/14	48/15	22/15	26/11

* Numerator=Number of fetuses affected; Denominator=Number of litters affected.

** Positive control: 250.0 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 171 & 172; 177 through 180 Date December 8, 1972

Material FDA 71-44 Laboratory No. 0902 y

Table 3-a

Summary of Soft Tissue Abnormalities

(Rats)

Group	Material	Dose Level mg/kg	Dam	Number of Pups	Description
172	Aspirin*	250.0	A 2511	1	Exencephaly
			A 2525	1	Exencephaly
			A 2534	3	Exencephaly
178	FDA 71-44	0.68	Y 2035	1	Short Tail

* Positive Control: 250.0 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 171 & 172; 177 through 180

Date December 8, 1972

Table 4

Laboratory No. 0902 y

Species Rats

Average Body Weights*

Group	Material	Dose Level	Day 0	6	11	15	20**
		mg/kg	g				
171	Sham	0.0	206	225	241	257	319 (23)
172	Aspirin***	250.0	211	230	239	252	307 (20)
177	FDA 71-44	0.23	201	221	237	253	315 (24)
178	FDA 71-44	0.68	211	228	243	253	324 (19)
179	FDA 71-44	3.2	199	217	231	250	313 (22)
180	FDA 71-44	22.5	207	227	236	247	303 (23)

* Of pregnant dams
** Number of surviving dams in parentheses (c.f. Table 1)
*** Positive control: 250.0 mg/kg

Appendix I

Teratology Study in Rats

Virgin adult female albino rats (Wistar derived stock) were individually housed in mesh bottom cages in temperature and humidity-controlled quarters with free access to food and fresh tap water. They were mated with young adult males, and observation of the vaginal sperm plug was considered Day 0 of gestation. Beginning on Day 6 and continuing daily through Day 15 of gestation, the females were dosed with the indicated dosages by oral intubation; the controls were sham treated.

Body weights were recorded on Days 0, 6, 11, 15, and 20 of gestation. All animals were observed daily for appearance and behavior with particular attention to food consumption and weight, in order to rule out any abnormalities which may have occurred as a result of anorexic effects in the pregnant female animal.

On Day 20 all dams were subjected to Caesarean section under surgical anesthesia, and the numbers of implantation sites, resorption sites, and live and dead fetuses were recorded. The body weights of the live pups were also recorded. The urogenital tract of each dam was examined in detail for anatomical normality.

All fetuses were examined grossly for the presence of external congenital abnormalities. One-third of the fetuses of each litter underwent detailed visceral examinations employing 10X magnification. The remaining two-thirds were cleared in potassium hydroxide (KOH), stained with alizarin red S dye and examined for skeletal defects.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 171

Material Sham

Dose 0.0 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date December 8, 1972

Laboratory No. 0902

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
S 2511	P	14	13	13		8	5		4.08	
S 2512	P	14	13	13		6	7		3.75	
S 2513	P	13	13	13		5	8		4.11	
S 2514	P	10	10	10		5	5		4.15	
S 2515	P	13	13	13		8	5		3.14	
S 2516	P	12	13	10		5	5	3	3.92	
S 2517	P	10	10	10		5	5		3.82	
S 2518	P	11	11	11		2	9		3.77	
S 2519	P	14	14	14		7	7		3.35	
S 2520	P	9	9	8		5	3	1	3.69	
S 2521	P	13	9	9		3	6		3.54	
S 2522	P	7	7	7		3	4		3.72	
S 2523	P	10	10	10		7	3		3.43	
S 2524	P	11	11	11		7	4		3.77	
S 2525	P	8	7	7		2	5		3.11	
S 2526	P	12	11	10	1	7	3		3.80	
S 2527	NP	5	0						----	
S 2528	P	12	11	11		4	7		3.89	
S 2529	P	12	12	12		8	4		3.88	
S 2530	P	8	2	2		1	1		5.28	
S 2531	P	14	14	14		6	8		3.65	
S 2532	P	5	1	1		0	1		4.74	
S 2533	P	11	11	11		5	6		3.97	
S 2534	P	11	10	10		5	5		3.84	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 172

Material Aspirin

Dose 250.0 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date December 8, 1972

Laboratory No. 0902

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
A 2511	P	9	9	9		6	3		2.60	
A 2512	P	10	9					9	----	
A 2513	P	5	10	2		1	1	8	2.01	
A 2514	P	10	10	10		6	4		3.36	
A 2515	P	13	13	13		6	7		2.76	
A 2516	P	15	12	12		3	9		2.22	
A 2517	NP	0	0						----	
A 2518	P	8	5	5		1	4		2.51	
A 2519	P	9	7	6		2	4	1	2.87	
A 2520	NP	21	0						----	
A 2521	P	11	10	10		4	6		2.64	
A 2522	NP	1	0						----	
A 2523	P	11	11	11		5	6		2.37	
A 2524	P	12	10	6		1	5	4	2.11	
A 2525	P	11	11	11		5	6		2.36	
A 2526	P	13	13	13		6	7		2.85	
A 2527	P	14	14	14		5	9		2.89	
A 2528	P	11	10	10		6	4		2.27	
A 2529	P	10	10	10		4	6		4.55	
A 2530	P	11	11	11		5	6		4.07	
A 2531	P	12	9	9		4	5		2.79	
A 2532	P	11	12	10		7	3	2	3.02	
A 2533	NP	3	0						----	
A 2534	P	13	13	13		5	8		2.23	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 177

Material FDA 71-44

Dose 0.23 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date December 8, 1972

Laboratory No. 0902 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 2001	P	11	11	11		5	6		3.98	
Y 2002	P	10	10	8		5	3	2	3.91	
Y 2003	P	11	11	11		6	5		3.78	
Y 2004	P	10	10	10		5	5		4.43	
Y 2005	P	11	11	11		8	3		3.87	
Y 2006	P	9	9	9		3	6		3.59	
Y 2007	P	14	12	12		6	6		3.75	
Y 2008	P	14	13	13		6	7		5.43	
Y 2009	P	12	11	11		4	7		3.92	
Y 2010	P	14	14	14		3	11		3.75	
Y 2011	P	12	12	12		8	4		3.50	
Y 2012	P	8	8	8		5	3		3.83	
Y 2013	P	8	8	8		6	2		2.32	
Y 2014	P	7	7	7		3	4		2.79	
Y 2015	P	8	8	8		3	5		2.52	
Y 2016	P	12	12	12		7	5		3.52	
Y 2017	P	14	14	14		5	9		3.82	
Y 2018	P	15	14	14		7	7		3.85	
Y 2019	P	12	8	8		4	4		3.73	
Y 2020	P	9	6	6		3	3		4.14	
Y 2021	P	10	10	10		6	4		3.63	
Y 2022	P	10	10	10		5	5		3.55	
Y 2023	P	13	14	13		7	6	1	3.81	
Y 2024	P	12	12	12		5	7		3.71	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 178

Material FDA 71-44

Dose 0.68 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date December 8, 1972

Laboratory No. 0902 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 2031	P	10	10	10		6	4		3.81	
Y 2032	NP	1	0						----	
Y 2033	P	11	11	11		10	1		4.11	
Y 2034	P	9	9	9		4	5		4.02	
Y 2035	P	10	10	10		2	8		4.12	
Y 2036	P	8	8	8		2	6		3.56	
Y 2037	P	17	16	16		5	11		3.57	
Y 2038	P	9	8	7		5	2	1	3.85	
Y 2039	P	11	11	11		3	8		3.96	
Y 2040	P	12	12	12		6	6		3.87	
Y 2041	P	14	14	14		5	9		3.89	
Y 2042	P	12	11	11		4	7		3.53	
Y 2043	P	10	10	9		4	5	1	2.22	
Y 2044	P	10	10	10		4	6		2.51	
Y 2045	P	9	8	8		4	4		2.89	
Y 2046	NP	0	0						----	
Y 2047	NP	0	0						----	
Y 2048	NP	5	0						----	
Y 2049	NP	9	0						----	
Y 2050	P	16	17	15		7	8	2	3.63	
Y 2051	P	16	15	15		5	10		3.82	
Y 2052	P	11	11	11		7	4		3.07	
Y 2053	P	12	12	12		4	8		3.55	
Y 2054	P	13	13	13		5	8		3.88	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 179

Material FDA 71-44

Dose 3.2 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date December 8, 1972

Laboratory No. 0902 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 2061	P	14	14	14		6	8		3.49	
Y 2062	P	11	11	11		3	8		3.69	
Y 2063	P	15	15	15		8	7		3.73	
Y 2064	NP	5	0						----	
Y 2065	P	13	13	13		8	5		4.07	
Y 2066	P	14	13	12		4	8	1	3.95	
Y 2067	P	9	10	8		4	4	2	3.46	
Y 2068	P	13	12	12		5	7		3.75	
Y 2069	P	12	8	8		2	6		3.84	
Y 2070	P	13	11	11		6	5		5.01	
Y 2071	P	12	11	11		4	7		3.81	
Y 2072	P	10	10	10		6	4		3.70	
Y 2073	P	13	7	7		2	5		4.01	
Y 2074	P	11	11	11		7	4		5.07	
Y 2065	P	12	12	12		5	7		3.52	
Y 2076	NP	3	0						----	
Y 2077	P	12	11	11		5	6		3.98	
Y 2078	P	10	9	9		8	1		3.80	
Y 2079	P	12	12	10		6	4	2	3.79	
Y 2080	P	14	14	14		4	10		3.54	
Y 2081	P	13	13	12		5	7	1	3.71	
Y 2082	P	14	13	13		6	7		3.52	
Y 2083	P	10	10	10		3	7		4.00	
Y 2084	P	11	10	10		5	5		3.49	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 180

Material FDA 71-44

Dose 22.5 mg/kg

Appendix II

Reproduction Data in Rats (Individual)

Date December 8, 1972

Laboratory No. 0902 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 2091	P	10	7	7		2	5		3.93	
Y 2092	P	9	9	9		4	5		3.89	
Y 2093	NP	20	0						----	
Y 2094	NP	17	0						----	
Y 2095	NP	5	0						----	
Y 2096	NP	8	0						----	
Y 2097	P	11	9	9		7	2		3.76	
Y 2098	P	13	12	12		3	9		3.82	
Y 2099	P	14	13	13		6	7		3.86	
Y 2100	P	11	10	9		5	4	1	3.96	
Y 2101	P	10	9	9		5	4		4.12	
Y 2102	P	9	8	8		3	5		3.93	
Y 2103	P	9	9	9		4	5		6.06	
Y 2104	P	13	12	12		6	6		5.17	
Y 2105	P	9	9	8		4	4	1	3.76	
Y 2106	P	13	11	10		7	3	1	3.81	
Y 2107	NP	2	0						----	
Y 2108	P	13	13	13		7	6		3.61	
Y 2109	P	11	11	11		6	5		3.64	
Y 2110	P	12	11	11		4	7		3.79	
Y 2111	P	14	14	14		6	8		3.55	
Y 2112	P	11	13	12		8	4	1	3.83	
Y 2113	P	9	7	6		2	4	1	3.42	
Y 2114	P	14	13	13		6	7		4.17	
Y 2115	P	14	14	14		6	8		3.63	
Y 2116	P	10	9	9		2	7		3.61	
Y 2117	P	9	10	9	1	3	6		3.33	
Y 2118	P	11	10	10		5	5		4.00	

* P = Pregnant; NP = Not Pregnant

Food and Drug Research Laboratories
INCORPORATED

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FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date January 8, 1973

Laboratory No. 0903 v
Contract No. FDA 71-260

Sample: Fine white powdered material

Marking: FDA 71-44 (Caffeine)

Examination Requested: Teratologic evaluation of FDA 71-44 in hamsters

Procedure: See Appendix I

Results: See Tables 1 through 4 and Appendix II

Conclusion: Subject to reexamination in the light of later findings, the following is concluded:

"The administration of up to 30 mg/kg (body weight) of the test material to pregnant hamsters for 5 consecutive days had no clearly discernible effect on nidation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

Comment: Attention is called to the fact that this is the eighteenth of a series of reports which will be issued in accordance with the terms of the contract cited above. Eventually, a total of at least 42 compounds will have been tested in 21 pairs; each pair being run concurrently against one sham-treated control and one positive control group. Because of the inherent variability of biological data of the type dealt with here, the accumulation and pooling of sequential sets of control values will greatly enhance the statistical value of the findings and the ultimate reliability of the test results.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgaleidge

Kenneth Morgaleidge, Ph.D.
Vice President

This report is submitted for the exclusive use of the person, partnership, or corporation to whom it is addressed, and neither the report nor the name of these Laboratories nor of any members of its staff, may be used in connection with the advertising or sale of any product or process without written authorization.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups: 171 & 172; 177 through 180

Material: FDA 71-44

Date: December 8, 1972

Laboratory No.: 0903 y

Table 1

Fate Summary
(Hamsters)

Group	Material	Dose ** mg/kg	Total		Surviving at Term	
			Mated	Pregnant	Total	Pregnant[1]
171	Sham	0.0	24	21	24	21
172	Aspirin*	250.0	22	20	22	20
177	FDA 71-44	0.3	22	21	22	21
178	FDA 71-44	1.4	22	21	21	20
179	FDA 71-44	6.5	26	20	26	20
180	FDA 71-44	30.0	27	20	27	20

* Positive Control: 250.0 mg/kg
** Administered as a water solution; (sham group dosed with anhydrous corn oil)
1) Includes all dams examined at term

FOOD AND DRUG RESEA LABORATORIES, INC.

Group: 171 & 172; 177 through 180

Material: FDA 71-44

Table 2
Reproduction Data
(Hamsters)

Date: December 8, 1972

Laboratory No.: 0903 y

Group:	171	172	177	178	179	180
Dose (mg/kg):	Sham	Aspirin**	0.3	1.4	6.5	30.0
Pregnancies						
Total No.	21	20	21	21	20	20
Died or Aborted (before Day 14)	0	0	0	1	0	0
To term (on Day 14)	21	20	21	20	20	20
Corpora Lutea						
Total No.	289	280	265	250	283	291
Average/dam mated	12.0	12.7	12.0	11.4	10.9	10.8
Live Litters						
Total No.*	20	20	21	20	20	20
Implant Sites						
Total No.	262	248	258	223	237	220
Average/dam*	12.5	12.4	12.3	11.2	11.9	11.0
Resorptions						
Total No.*	10	3	2	1	0	6
Dams with 1 or more sites resorbed	4	3	1	1	--	5
Dams with all sites resorbed	--	--	--	--	--	--
Per cent partial resorptions	19.0	15.0	4.76	5.00	--	25.0
Per cent complete resorptions	--	--	--	--	--	--
Live Fetuses						
Total No.	232	243	254	222	232	213
Average/dam*	11.0	12.2	12.1	11.1	11.6	10.7
Sex ratio (M/F)	0.73	0.71	0.79	0.73	0.61	0.76
Dead Fetuses						
Total No.*	20	2	2	--	5	1
Dams with 1 or more dead	2	2	2	--	4	1
Dams with all dead	1	--	--	--	--	--
Per cent partial dead	9.52	10.0	9.52	--	20.0	5.00
Per cent all dead	4.76	--	--	--	--	--
Average Fetus Weight, g	1.71	1.71	1.73	1.84	1.82	1.79

* Includes only those dams examined at term.

** Positive Control: 250.0 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 171 & 172; 177 through 180 Date December 8, 1972

Material FDA 71-44 Laboratory No. 0903 y

Table 3-a

Summary of Soft Tissue Abnormalities

(Hamsters)

Group	Material	Dose Level mg/kg	Dam	Number of Pups	Description
172	Aspirin*	250.0	A 3514	1	Club foot
			A 3520	1	Meningoencephalocele
177	FDA 71-44	0.3	Y 3003	2	Club foot

* Positive Control: 250.0 mg/kg

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Date December 8, 1972

Groups 171 & 172; 177 through 180

Table 4

Laboratory No. 0903 y

Species Hamsters

Average Body Weights*

Group	Material	Dose Level	Day 0	6	8	10	14**
		mg/kg	g				
171	Sham	0.0	103.4	109.9	113.7	123.5	146.8 (21)
172	Aspirin***	250.0	107.3	117.7	118.4	132.5	152.4 (20)
177	FDA 71-44	0.3	109.2	118.5	120.5	131.0	152.2 (21)
178	FDA 71-44	1.4	96.0	105.1	108.2	118.6	136.7 (20)
179	FDA 71-44	6.5	97.5	103.9	111.1	121.9	144.4 (20)
180	FDA 71-44	30.0	95.2	103.1	107.6	116.9	136.4 (20)

* Of pregnant dams
** Number of surviving dams in parentheses (c.f. Table 1)
*** Positive control: 250.0 mg/kg

Appendix I

Teratology Study in Hamsters

Virgin adult female golden hamsters from an outbred strain were individually housed in mesh bottom cages in temperature and humidity controlled quarters with free access to food and fresh tap water at all times. They were mated (1 to 1) with mature males and the appearance of motile sperm in the vaginal smear was considered as Day 0 of gestation. Beginning on Day 6 and continuing daily through Day 10 of gestation, the indicated dose levels of the test material were administered by oral intubation; the controls were sham-treated.

Body weights were recorded on Days 0, 8, 10, and 14 of the gestation period. All animals were observed daily for appearance and behavior with particular attention to food consumption in order to better recognize any abnormalities resulting from anorexic effects in the pregnant animal.

On Day 14, all animals were subjected to Caesarian section under deep anesthesia and the numbers of implantation sites, resorption sites, live and dead fetuses were recorded. All live pups were weighed and the genital tract of each dam was examined for any anatomical abnormalities.

All fetuses were examined grossly for the presence of external congenital defects and one-third of each litter underwent detailed visceral examination under 10X magnification. The remaining two-thirds of the pups were cleared in potassium hydroxide, stained with alizarin red dye, and examined for the presence of sketal abnormalities.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Date December 8, 1972

Group 171

Appendix II

Laboratory No. 0903

Material Sham

Reproduction Data in Hamsters (Individual)

Dose 0.0 mg/kg

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
S 3511	P	10	11	10		6	4	1	1.63	
S 3512	P	9	11	9		3	6	2	1.61	
S 3513	P	10	10	10		5	5		2.01	
S 3514	P	21	17		17				----	
S 3515	NP	3	0						----	
S 3516	P	14	14	14		6	8		1.71	
S 3517	P	12	12	12		6	6		1.72	
S 3518	P	10	9	9		3	6		1.81	
S 3519	P	12	11	11		6	5		1.85	
S 3520	P	17	15	15		4	11		1.82	
S 3521	P	15	15	15		7	8		1.63	
S 3522	P	12	13	7		3	4	6	1.35	
S 3523	P	13	13	12		3	9	1	1.38	
S 3524	P	14	14	14		2	12		1.50	
S 3525	NP	12	0						----	
S 3526	P	11	10	10		7	3		1.71	
S 3527	P	10	10	10		6	4		1.89	
S 3528	P	11	11	11		4	7		1.90	
S 3529	P	14	14	11	3	6	5		1.75	
S 3530	NP	4	0						----	
S 3531	P	14	14	14		6	8		1.74	
S 3532	P	14	14	14		5	9		1.79	
S 3533	P	13	11	11		6	5		1.79	
S 3534	P	14	13	13		4	9		1.69	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Appendix II

Reproduction Data in Hamsters (Individual)

Group 172

Material Aspirin

Dose 250.0 mg/kg

Date December 8, 1972

Laboratory No. 0903

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
A 3511	P	10	10	10		4	6		1.76	
A 3512	P	9	9	8		2	6	1	1.75	
A 3513	P	18	18	18		7	11		1.59	
A 3514	P	14	14	14		5	9		0.96	
A 3515	P	16	13	13		4	9		1.79	
A 3516	P	14	11	11		5	6		1.89	
A 3517	P	19	14	13		6	7	1	1.77	
A 3518	NP	7	0						----	
A 3519	P	10	10	10		5	5		1.83	
A 3520	P	11	8	7	1	3	4		1.88	
A 3521	P	18	18	18		8	10		1.53	
A 3522	P	18	17	16	1	6	10		1.72	
A 3523	P	10	10	10		2	8		1.85	
A 3524	NP	0	0						----	
A 3525	P	14	14	14		8	6		1.49	
A 3526	P	11	10	10		5	5		1.75	
A 3527	P	11	11	10		3	7	1	1.49	
A 3528	P	15	11	11		5	6		1.79	
A 3529	P	13	13	13		4	9		1.61	
A 3530	P	16	12	12		[illegible]	4		1.86	
A 353[illegible]	P	13	13	13		4	9		1.73	
A 3532	P	13	12	12		[illegible]	6		2.09	

* P = Pregnant NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Date December 8, 1972

Laboratory No. 0903 y

Group 177

Material FDA 71-44

Dose 0.3 mg/kg

Appendix II

Reproduction Data in Hamsters (Individual)

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 3001	P	11	12	10		5	5	2	1.71	
Y 3002	P	10	9	9		3	6		1.76	
Y 3003	P	15	15	15		6	9		0.92	
Y 3004	P	16	16	16		8	8		1.76	
Y 3005	P	13	13	13		6	7		1.79	
Y 3006	P	12	12	12		6	6		1.84	
Y 3007	P	12	12	12		7	5		1.86	
Y 3008	P	12	13	13		7	6		1.68	
Y 3009	P	12	11	11		5	6		1.80	
Y 3010	P	16	16	15	1	11	4		1.64	
Y 3011	NP	3	0						----	
Y 3012	P	9	9	9		1	8		1.66	
Y 3013	P	15	14	14		6	8		1.80	
Y 3014	P	12	11	11		2	9		1.79	
Y 3015	P	11	11	11		4	7		1.91	
Y 3016	P	12	12	12		3	9		1.92	
Y 3017	P	12	11	11		6	5		1.64	
Y 3018	P	14	14	14		8	6		1.98	
Y 3019	P	14	14	13	1	5	8		1.55	
Y 3020	P	12	12	12		6	6		1.67	
Y 3021	P	12	12	12		3	9		1.[illegible]2	
Y 3022	P	10	9	9		4	5		1.[illegible]4	

* P = Pregnant; [illegible]

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Appendix II

Reproduction Data in Hamsters (Individual)

Group 178

Material FDA 71-44

Dose 1.4 mg/kg

Date December 8, 1972

Laboratory No. 0903 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 3031	P	12	12	12		3	9		----	Aborted Day 14
Y 3032	P	13	13	13		7	6		1.98	
Y 3033	NP	0	0						----	
Y 3034	P	13	13	13		7	6		1.75	
Y 3035	P	12	12	12		5	7		2.03	
Y 3036	P	11	9	9		4	5		1.88	
Y 3037	P	10	10	10		3	7		1.92	
Y 3038	P	9	7	7		3	4		1.93	
Y 3039	P	10	10	10		4	6		2.08	
Y 3040	P	15	15	15		7	8		1.81	
Y 3041	P	13	11	11		5	6		1.72	
Y 3042	P	18	15	15		7	8		1.78	
Y 3043	P	10	9	8		5	3	1	1.55	
Y 3044	P	10	10	10		5	5		2.10	
Y 3045	P	13	12	12		3	9		1.79	
Y 3046	P	11	11	11		4	7		1.75	
Y 3047	P	13	10	10		4	6		1.66	
Y 3048	P	12	11	11		4	7		1.79	
Y 3049	P	13	13	13		5	8		1.72	
Y 3050	P	10	10	10		4	6		1.87	
Y 3051	P	12	12	12		4	8		1.63	
Y 3052	P	10	10	10		4	6		1.96	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 179

Material FDA 71-44

Dose 6.5 mg/kg

Appendix II

Reproduction Data in Hamsters (Individual)

Date December 8, 1972

Laboratory No. 0903 Y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 3061	P	10	10	8	2	4	5		1.54	
Y 3062	P	12	12	11	1	3	8		1.99	
Y 3063	P	19	18	18		8	10		1.67	
Y 3064	P	12	11	11		5	6		2.08	
Y 3065	P	12	12	12		4	8		1.91	
Y 3066	P	11	10	10		4	6		2.06	
Y 3067	NP	3	0						----	
Y 3068	NP	5	0						----	
Y 3069	P	15	14	14		6	8		1.97	
Y 3070	P	10	9	9		5	4		2.01	
Y 3071	P	16	16	16		4	12		1.83	
Y 3072	NP	7	0						----	
Y 3073	P	11	9	9		4	5		1.71	
Y 3074	P	9	9	9		4	5		1.67	
Y 3075	P	12	11	11		5	6		1.85	
Y 3076	P	12	12	12		5	7		1.87	
Y 3077	P	14	13	13		5	8		1.81	
Y 3078	NP	11	0						----	
Y 3079	NP	3	0						----	
Y 3080	P	13	12	11	1	3	8		1.76	
Y 3081	P	12	12	12		5	7		1.96	
Y 3082	P	14	14	13	1	4	[illegible]		1.8[illegible]	
Y 3083	P	13	9	9		2	7		1.[illegible]	
Y 3084	P	11	11	11		4	7		1.70	
Y 3085	NP	2	0						----	
Y 3086	P	14	13	13		4	9		1.56	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 180

Material FDA 71-44

Dose 30.0 mg/kg

Appendix II

Reproduction Data in Hamsters (Individual)

Date December 8, 1972

Laboratory No. 0903 Y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 3091	P	6	9	8		5	3	1	1.71	
Y 3092	P	12	12	12		4	8		1.71	
Y 3093	P	16	10	9		6	3	1	1.55	
Y 3094	NP	8	0						----	
Y 3095	P	10	10	10		6	4		1.93	
Y 3096	P	10	10	10		5	5		1.69	
Y 3097	P	13	13	12		5	7	1	1.67	
Y 3098	NP	9	0						----	
Y 3099	P	12	12	12		6	6		1.86	
Y 3100	P	10	2	2		1	1		2.18	
Y 3101	P	12	11	11		5	6		1.79	
Y 3102	P	14	13	13		7	6		1.73	
Y 3103	NP	8	0						----	
Y 3104	P	10	10	10		3	7		1.67	
Y 3105	P	10	11	8	1	3	5	2	1.73	
Y 3106	P	10	10	10		6	4		1.91	
Y 3107	NP	2	0						----	
Y 3108	NP	3	0						----	
Y 3109	P	13	12	12		2	10		1.75	
Y 3110	NP	11	0						----	
Y 3111	P	14	12	12		3	9		1.80	
Y 3112	P	13	13	13		3	10		1.84	
Y 3113	P	14	14	13		4	9	1	1.67	
Y 3114	P	12	10	10		4	6		1.87	
Y 3115	NP	4	0						----	
Y 3116	P	18	13	13		8	5		1.78	
Y 3117	P	15	13	13		6	7		1.72	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 179

Material FDA 71-44

Dose 6.5 mg/kg

Appendix II

Reproduction Data in Hamsters (Individual)

Date December 8, 1972

Laboratory No. 0903 Y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 3061	P	10	10	8	2	4	5		1.54	
Y 3062	P	12	12	11	1	3	8		1.99	
Y 3063	P	19	18	18		8	10		1.67	
Y 3064	P	12	11	11		5	6		2.08	
Y 3065	P	12	12	12		4	8		1.91	
Y 3066	P	11	10	10		4	6		2.06	
Y 3067	NP	3	0						----	
Y 3068	NP	5	0						----	
Y 3069	P	15	14	14		6	8		1.97	
Y 3070	P	10	9	9		5	4		2.01	
Y 3071	P	16	16	16		4	12		1.83	
Y 3072	NP	7	0						----	
Y 3073	P	11	9	9		4	5		1.71	
Y 3074	P	9	9	9		4	5		1.67	
Y 3075	P	12	11	11		5	6		1.85	
Y 3076	P	12	12	12		5	7		1.87	
Y 3077	P	14	13	13		5	8		1.81	
Y 3078	NP	11	0						----	
Y 3079	NP	3	0						----	
Y 3080	P	13	12	11	1	3	8		1.76	
Y 3081	P	12	12	12		5	7		1.96	
Y 3082	P	14	14	13	1	4	9		1.86	
Y 3083	P	13	9	9		2	7		1.51	
Y 3084	P	11	11	11		4	7		1.70	
Y 3085	NP	2	0						----	
Y 3086	P	14	13	13		4	9		1.56	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 180

Material FDA 71-44

Dose 30.0 mg/kg

Appendix II

Reproduction Data in Hamsters (Individual)

Date December 8, 1972

Laboratory No. 0903 Y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 3091	P	8	9	8		5	3	1	1.71	
Y 3092	P	12	12	12		4	8		1.71	
Y 3093	P	16	10	9		6	3	1	1.55	
Y 3094	NP	8	0						----	
Y 3095	P	10	10	10		6	4		1.93	
Y 3096	P	10	10	10		5	5		1.69	
Y 3097	P	13	13	12		5	7	1	1.87	
Y 3098	NP	9	0						----	
Y 3099	P	12	12	12		6	6		1.86	
Y 3100	P	10	2	2		1	1		2.18	
Y 3101	P	12	11	11		5	6		1.79	
Y 3102	P	14	13	13		7	6		1.73	
Y 3103	NP	8	0						----	
Y 3104	P	10	10	10		3	7		1.67	
Y 3105	P	10	11	8	1	3	5	2	1.73	
Y 3106	P	10	10	10		6	4		1.91	
Y 3107	NP	2	0						----	
Y 3108	NP	3	0						----	
Y 3109	P	13	12	12		2	10		1.75	
Y 3110	NP	11	0						----	
Y 3111	P	14	12	12		3	9		1.80	
Y 3112	P	13	13	13		3	10		1.84	
Y 3113	P	14	14	13		4	9	1	1.67	
Y 3114	P	12	10	10		4	6		1.87	
Y 3115	NP	4	0						----	
Y 3116	P	18	13	13		8	5		1.78	
Y 3117	P	15	13	13		6	7		1.72	

* P = Pregnant; NP = Not Pregnant

FDRL

FOOD AND DRUG Research LABORATORIES, INC.

WAVERLY DIVISION
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FINAL REPORT

Submitted to: DHEW/Public Health Service
Food and Drug Administration CA-272
5600 Fishers Lane-Room 5C-13
Rockville, Maryland 20852

Date July 31, 1973

Laboratory No. 0904 y
Contract No. FDA 71-260

Sample: Fine white powdered material

Marking: FDA 71-44 (Caffeine)

Examination Requested: Teratologic evaluation of FDA 71-44 in rabbits

Procedure: (See Appendix I)

Results: See Tables 1 through 4 and Appendix II

Conclusion:

On the basis of the data presented herein, the following conclusion appears to be warranted:

"The administration of up to 7.0 mg/kg (body weight) of the test material to pregnant rabbits for 13 consecutive days had no clearly discernible effect on nidation or on maternal or fetal survival. The number of abnormalities seen in either soft or skeletal tissues of the test groups did not differ from the number occurring spontaneously in the sham-treated controls."

However, at the highest dose level (32.5 mg/kg), there appeared to be an increase in maternal mortality with a decrease in the fertility rate. There was no clear indication that caffeine was teratogenic even at this level.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Kenneth Morgareidge

Kenneth Morgareidge, Ph.D.
Vice President

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FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups: 171 & 172; 177 through 180

Material: FDA 71-44

Date: June 28, 1973

Laboratory No.: 0904 y

Table 1

Fate Summary

(Rabbits)

Group	Material	Dose** mg/kg	Total		Surviving at Term	
			Mated	Pregnant	Total	Pregnant[1]
171	Sham	0.0	14	8	12	7
172	6-AN*	2.5	14	9	12	8
177	FDA 71-44	0.325	24	10	20	8
178	FDA 71-44	1.5	23	13	21	11
179	FDA 71-44	7.0	15	11	14	10
180	FDA 71-44	32.5	30	19	24	13

* Positive Control: 2.5 mg/kg of 6-aminonicotinamide dosed on Day 9

** Administered as a water solution (See Appendix I)

1) Includes all dams examined at term

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group: 171 & 172; 177 through 180

Material: FDA 71-44

Table 2
Reproduction Data
(Rabbits)

Date: June 28, 1973

Laboratory No.: 0904 y

Group:	171	172	177	178	179	180
Dose (mg/kg):	Sham	6-AN**	0.325	1.5	7.0	32.5
Pregnancies						
Total No.	8	9	10	13	11	19
Died or Aborted (before Day 29)	2	1	2	2	1	6
To term (on Day 29)	7	8	8	11	10	13
Corpora Lutea						
Total No.	221	199	262	293	216	409
Average/dam mated	15.8	14.2	10.9	12.7	14.4	13.6
Live Litters						
Total No.*	6	6	7	10	9	9
Implant Sites						
Total No.	47	36	45	68	61	61
Average/dam*	6.71	4.50	5.63	6.18	6.10	4.69
Resorptions						
Total No.*	19	7	11	18	12	23
Dams with 1 or more sites resorbed	4	4	5	6	6	8
Dams with all sites resorbed	1	2	--	1	1	4
Per cent partial resorptions	57.1	50.0	62.5	54.6	60.0	61.5
Per cent complete resorptions	14.3	25.0	--	9.09	10.0	30.8
Live Fetuses						
Total No.	28	29	32	50	48	38
Average/dam*	4.00	3.63	4.00	4.55	4.80	2.92
Sex ratio (M/F)	0.87	0.93	0.78	0.96	0.85	1.00
Dead Fetuses						
Total No.*	--	--	2	--	1	--
Dams with 1 or more dead	--	--	2	--	1	--
Dams with all dead	--	--	--	--	--	--
Per cent partial dead	--	--	25.0	--	10.0	--
Per cent all dead	--	--	--	--	--	--
Average Fetus Weight, g	35.6	34.9	38.5	39.6	41.2	38.8

* Includes only those dams examined at term.

** Positive Control: 2.5 mg/kg of 6-aminonicotinamide dosed on Day 9

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 171 & 172; 177 through 180 Laboratory No. 0904 y

Table 3

Material FDA 71-44 Date June 28, 1973

Summary of Skeletal Findings*

(Rabbits)

Findings / Group No.:	171	172	177	178	179	180
Dose (mg/kg):	Sham	6-AN**	0.325	1.5	7.0	32.5
Live Fetuses Examined (at term)	28/6	29/6	32/7	50/10	48/9	34/8[a]
Sternebrae						
Incomplete oss.		1/1	2/1	1/1	2/2	2/1
Scrambled						
Bipartite		2/2				3/2
Fused	2/1	1/1	3/3	1/1		1/1
Extra	1/1			1/1	1/1	
Missing	1/1					
Other						
Ribs						
Incomplete oss.						
Fused/split		7/3				
Wavy						
Less than 12						
More than 13		5/1				
Other						
Vertebrae						
Incomplete oss.		8/2		1/1		1/1
Scrambled			1/1			
Fused						
Extra ctrs. oss.						
Scoliosis		6/2				
Tail defects		12/3		1/1		
Other		1/1				
Skull						
Incomplete closure	1/1		1/1		3/1	
Missing						
Craniostosis		3/1				2/1
Other						
Extremities						
Incomplete oss.						
Missing						
Extra						
Miscellaneous						

* Numerator=Number of fetuses affected; Denominator=Number of litters affected.

** Positive control: 2.5 mg/kg of 6-aminonicotinamide dosed on Day 9

a One litter lost in processing

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 171 & 172; 177 through 180 Date June 28, 1973

Material FDA 71-44 Laboratory No. 0904 y

Table 3-a
Summary of Soft Tissue Abnormalities
(Rabbits)

Group	Material	Dose Level mg/kg	Dam	Number of Pups	Description
171	Sham	0.0	S 4262	1	Encephalocele
172	6-AN*	2.5	Z 4264	1	Cleft palate
				1	Medial rotation; club foot; cleft palate
				1	Medial rotation; short tail
			Z 4267	4	Encephalocele; anopia; club foot
				1	Anopia; club foot; short tail
				1	Anopia; short tail

* Positive Control: 2.5 mg/kg of 6-aminonicotinamide dosed on Day 9

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Groups 171 & 172; 177 through 180

Date June 28, 1973

Table 4

Species Rabbits

Laboratory No. 0904 y

Average Body Weights*

Group	Material	Dose Level	Day 0	Day 6	Day 12	Day 18	Day 29**
		mg/kg	kg				
171	Sham	0.0	2.04	2.11	2.15	2.20	2.26 (7)
172	6-AN***	2.5	2.23	2.26	2.30	2.42	2.53 (8)
177	FDA 71-44	0.325	2.05	2.12	2.15	2.22	2.28 (8)
178	FDA 71-44	1.5	2.39	2.33	2.42	2.47	2.61 (11)
179	FDA 71-44	7.0	2.18	2.23	2.28	2.41	2.44 (10)
180	FDA 71-44	32.5	2.12	2.15	2.18	2.23	2.32 (13)

* Of pregnant dams
** Number of surviving dams in parentheses (c.f. Table 1)
*** Positive control: 2.5 mg/kg of 6-aminonicotinamide dosed on Day 9

Appendix I

Teratology Study in Rabbits

Virgin, adult, Dutch-belted female rabbits were individually housed in mesh bottom cages in temperature and humidity-controlled quarters with free access to food and fresh tap water. On Day 0, each doe was given an injection of 0.4 ml of human chorionic gonadotropin (400 IU) via the marginal ear vein. Three hours later, each doe was inseminated artificially with 0.3 ml of diluted semen from a proven donor buck using approximately 20×10^6 motile sperm according to the procedure described by Vogin et al (Pharmacologist 11, 282 (1969)). Beginning on Day 6 and continuing daily through Day 18 the females were dosed with the indicated dosages by oral intubation. The controls were sham treated with the vehicle at a level equivalent to the group receiving the highest test dose. The test material was prepared and doses calculated according to the following table:

Dosage (mg/kg)	Dose (ml/kg)	Concentration (mg/ml)
≦ 250	1	≦ 250
251 - 500	2	125 - 250
501 - 750	3	133 - 250
751 - 1000	4	187 - 250
1001 - 1250	5	200 - 250
1251 - 1500	6	208 - 250
1501 - 1600	6.4	235 - 250

Body weights were recorded on Days 0, 6, 12, 18, and 29 of gestation. All animals were observed daily for appearance and behavior, with particular attention to food consumption and body weight in order to rule out any abnormalities which may have occurred as a result of anorexic effects in the pregnant female animal.

On Day 29 all does were subjected to Caesarean section under surgical anesthesia, and the numbers of corpora lutea, implantation sites, resorption sites and live and dead fetuses were recorded. Body weights of the live pups were also recorded. The urogenital tract of each animal was examined in detail for normality. In addition all fetuses underwent a detailed gross examination for the presence of external congenital abnormalities. The live fetuses of each litter were then placed in an incubator for 24 hours for the evaluation of neonatal survival. All surviving pups were sacrificed, and all pups examined for visceral abnormalities (by dissection). All fetuses were then cleared in potassium hydroxide (KOH), stained with alizarin red S dye and examined for skeletal defects.

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Appendix II

Reproduction Data in Rabbits (Individual)

Group 171

Material Sham

Dose 0.0 mg/kg

Date June 28, 1973

Laboratory No. 0904

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
S 4256	P	17	8	4		1	3	4	32.9	
S 4257	NP	4	0						----	
S 4258	NP	10	0						----	
S 4259	NP	7	0						----	
S 4260	NP	9	0						----	
S 4261	P	19	9					9	----	
S 4262	P	36	9	9		4	5		31.4	
S 4263	NP	7	0						----	Died Day 21
S 4264	P	11	3	--		--	--		----	Died Day 26
S 4265	P	24	5	3		1	2	2	34.2	
S 4266	P	28	5	1		1	0	4	37.3	
S 4267										Not Assigned
S 4268	P	24	8	8		5	3		33.4	
S 4269	NP	3	0						----	
S 4270	P	22	3	3		1	2		44.3	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 172

Material 6-AN

Dose 2.5 mg/kg

Appendix II

Reproduction Data in Rabbits (Individual)

Date June 28, 1973

Laboratory No. 0904

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Z 4256	P	19	5	5		3	2		38.5	
Z 4257	NP	17	0						----	
Z 4258	NP	12	0						----	Died Day 16
Z 4259	P	18	5	5		2	3		36.0	
Z 4260	NP	12	0						----	
Z 4261	P	13	6	5		2	3	1	31.0	
Z 4262	P	9	1					1	----	
Z 4263	NP	6	0						----	
Z 4264	P	23	7	3		2	1	4	31.3	Three Neonatal De
Z 4265										Not Assigned
Z 4266	P	23	8	--		--	--		----	Died Day 22
Z 4267	P	14	6	6		3	3		35.7	Four Neonatal Dea
Z 4268	NP	7	0						----	
Z 4269	P	18	5	5		2	3		36.8	
Z 4270	P	8	1					1	----	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 177

Material FDA 71-44

Dose 0.325 mg/kg

Appendix II

Reproduction Data in Rabbits (Individual)

Date June 28, 1973

Laboratory No. 0904 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 4001	P	17	7	5		2	3	2	34.2	
Y 4002	NP	10	0						----	
Y 4003	P	9	3	1		0	1	2	49.1	
Y 4004	P	13	7	7		2	5		24.4	Two Neonatal Deat
Y 4005	P	13	2	2		1	1		51.1	
Y 4006	NP	0	0						----	
Y 4007	NP	6	0						----	
Y 4008	NP	4	0						----	Died Day 11
Y 4009	NP	2	0						----	
Y 4010	NP	3	0						----	
Y 4011	NP	7	0						----	
Y 4012	NP	3	0						----	
Y 4013	NP	10	0						----	
U 4014	NP	2	0						----	
Y 4015	NP	7	0						----	
Y 4061	P	16	7	6		3	3	1	45.6	
Y 4062	P	21	6		1			5	----	
Y 4063	P	35	6	5	1	2	3		33.0	
Y 4064	NP	12	0						----	
Y 4065	P	21	7	6		4	2	1	32.4	
Y 4082	P	16	6	--		--	--		----	Died Day 11
Y 4083	NP	4	0						----	Died Day 13
Y 4084	P	23	3	--		--	--		----	Accid. Death Day
Y 4085	NP	8	0						----	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 178

Material FDA 71-44

Dose 1.5 mg/kg

Appendix II

Reproduction Data in Rabbits (Individual)

Date June 28, 1973

Laboratory No. 0904 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 4016	P	8	6					6	----	
Y 4017	NP	3	0						----	
Y 4018	P	21	7	5		4	1	2	40.2	
Y 4019	P	24	6	6		3	3		37.6	
Y 4020	P	26	8	4		1	3	4	41.4	
Y 4021	NP	1	0						----	
Y 4022	NP	9	0						----	
Y 4023	NP	4	0						----	
Y 4024	P	29	11	--		--	--		----	Died Day 5
Y 4025	P	21	5	5		1	4		39.9	One Neonatal Death
Y 4026	P	25	6	3		--**	--**	3	30.6	
Y 4027	NP	5	0						----	
Y 4028	P	27	7	7		4	3		47.2	Two Neonatal Deaths
Y 4029	P	16	5	4		2	2	1	34.0	One Neonatal Death
Y 4030	NP	0	0						----	
Y 4066	NP	0	0						----	
Y 4067	NP	2	0						----	
Y 4068	NP	8	0						----	
Y 4069	NP	4	0						----	
Y 4086	P	14	7	5		1	4	2	26.6	
Y 4087	P	15	7	--		--	--		----	Died Day 13
Y 4088	P	14	5	5		3	2		49.1	
Y 4089	P	17	6	6		4	2		49.2	

* P = Pregnant; NP = Not Pregnant
** Not Recorded

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Appendix II

Reproduction Data in Rabbits (Individual)

Group 179

Material FDA 71-44

Dose 7.0 mg/kg

Date June 28, 1973

Laboratory No. 0904 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 4031	P	12	5	--		--	--		----	Died Day 17
Y 4032	P	22	9	5		3	2	4	36.6	One Neonatal Deat
Y 4033	P	18	7	6		3	3	1	39.7	
Y 4034	P	16	6	5	1	1	4		46.4	Two Neonatal Deat
Y 4035	P	24	8	6		3	3	2	39.3	
Y 4036	P	26	7	6		3	3	1	35.3	
Y 4037	P	23	4	4		2	2		44.2	
Y 4038	P	12	3					3	----	
Y 4039	P	18	6	5		2	3	1	42.6	
Y 4040	P	16	5	5		2	3		45.3	
Y 4041	NP	9	0						----	
Y 4042	NP	3	0						----	
Y 4043	NP	0	0						----	
Y 4044	NP	1	0						----	
Y 4045	P	16	6	6		3	3		41.5	

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Date June 28, 1973

Group 180

Appendix II

Laboratory No. 0904 y

Material FDA 71-44

Reproduction Data in Rabbits (Individual)

Dose 32.5 mg/kg

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Y 4046	NP	10	0						----	
Y 4047	P	5	3					3	----	
Y 4048	P	50	5	--		--	--		----	Died Day 17
Y 4049	NP	7	0						----	
Y 4050	NP	11	0						----	
Y 4051	NP	7	0						----	
Y 4052	NP	10	0						----	
Y 4053	P	22	3	3		1	2		46.6	
Y 4054	NP	10	0						----	
Y 4055	P	12	3					3	----	
Y 4056	P	18	4	3		2	1	1	38.5	
Y 4057	NP	5	0						----	
Y 4058	NP	5	0						----	
Y 4059	NP	9	0						----	
Y 4060	NP	1	0						----	
Y 4070	P	24	8	--		--	--		----	Died Day 17
Y 4071	NP	2	0						----	
Y 4072	P	14	6	--		--	--	1	----	Died Day 25
Y 4073	P	17	4	2		2	0	2	43.4	
Y 4074	P	7	6	6		2	4		31.3	Six Neonatal De
Y 4075	P	10	6	1		1	0	5	50.0	
Y 4076	P	9	7	7		4	3		27.2	Two Neonatal De
Y 4077	P	10	8	8		4	4		32.0	Two Neonatal De
Y 4078	P	32	8	7		--	--	1	----	Sacrificed Day
Y 4079	P	15	1					1	----	
Y 4080	P	12	6	5		2	3	1	41.1	One Neonatal De

Continued on next page

* P = Pregnant; NP = Not Pregnant

FOOD AND DRUG RESEARCH LABORATORIES, INC.

Group 180

Material FDA 71-44

Dose 32.5 mg/kg

Appendix II

Reproduction Data in Rabbits (Individual)

Date June 28, 1973

Laboratory No. 0904 y

Dam No.	Fate*	Corpora Lutea	Implant Sites	Fetuses Alive	Fetuses Dead	Sex M	Sex F	Resorption Sites	Average Fetus Weight (g)	Remarks
Continued										
Y 4081	P	22	4	--		--	--		----	Died Day 13
Y 4090	P	10	3	3		1	2		39.3	
Y 4091	P	24	7	6		--	--	1	----	Died Day 9
Y 4092	P	19	7					7	----	

* P = Pregnant; NP = Not Pregnant

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X-RAY EXAMINATIONS OF THE PASSAGE OF CAFFEINE IN DOGS

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The secretory effect of caffeine on the gastric glands of both man and animals is perfectly clear, while the most contrary data are encountered concerning observations of motility, peristalsis and tone conditions in man as also in animal experiments. The pharmacology of purine derivatives has been dealt with by *Bock* (1) in a most elaborate paper which, however, fails to offer satisfactory information on the subject, nor has our knowledge in this line been enriched during the period of over 30 years elapsed since.

It is recognized that caffeine is absorbed from the stomach and its action lasts 5—6 hours. According to *Issekutz* (6), the effect it exerts on the smooth muscle elements is manifested by increased peristalsis and consequent diarrhoea. *Poulsson-Liljestrand* (8) describe similar findings in their book. Relying on the experiments of *Walters* et al., *Goodman—Gilman* (5) maintain that in isolated intestinal loops dilute solutions augment, concentrated solutions diminish the number and amplitude of contractions, whereas in vivo the therapeutic doses of the xanthines show no marked influence on the motility of the gastro-intestinal canal. *Walters* et al. assert that in man theophylline aethylendiamine is capable of relieving in the bile duct the spasms caused by morphine and its derivatives. On the strength of this observation they recommend xanthines for the treatment of biliary colic. According to *Starkenstein* (9), caffeine as well as theophylline, accelerate the pendulating and caterpillar-like motion of the guinea pig's small intestine, raising its tone. In situ preparations of caffeine administered intravenously exert a stimulant effect on the small intestines of rabbits. This action is especially marked where intestinal function is inhibited by adrenaline or papaverine.

Mozgov (7) deals in his veterinary pharmacology with the effect of caffeine on the smooth muscle elements with special regard to the horse. He expresses his opinion that caffeine regulates the motoric functions of the alimentary tract through the vagus nerve centre. In some cases of colic affections it alleviates pain.

Our investigations of the passage of caffeine in dogs have been carried out within the series of pharmacodynamic investigations reports on which have been published in various periodicals [2, 3, 4]. In our research work we concentrated on the study of passage time in untreated dogs and the influence of certain drugs on the progress of the stomach and bowel contents through the gastro-intestinal tract.

Our examinations were performed on 4 dogs each weighing 10 kg and accustomed to similar experiments. Thanks to the approximately uniform results of the tests, it was unnecessary to raise the number of laboratory animals

but each experiment was repeated several times. Caffeine was given in four kinds of dosage administered orally, subcutaneously or intravenously. In the first series we gave 0,10 g of caffeine subcutaneously, in the second series black coffee containing about 0,20 g caffeine was mixed to the contrast medium and introduced through a tube; in the third series 0,25 g, in the fourth 0,50 g caffeine was injected intravenously.

For our experiments we used caffeine benzoic sodium ampoules. Of each series of tests we took 5 radiographs; analysis of the pictures, however, enabled

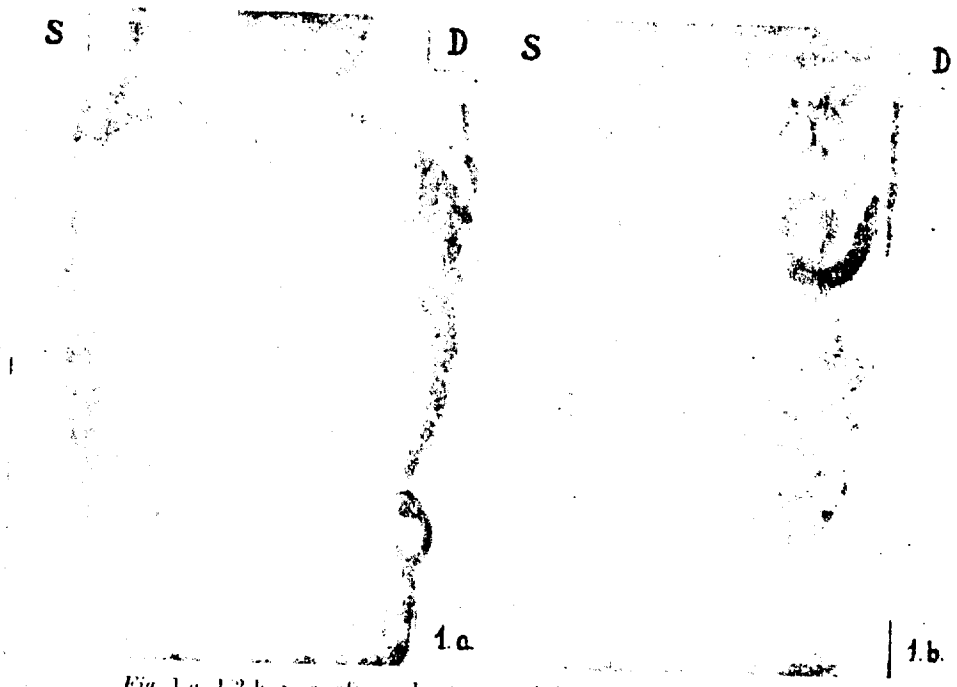

Fig. 1.*a.* 1/2 h. p. c. after subcutaneous injection of 0,10 g of caffeine
Fig. 1.*b.* 4 h. p. c. after subcutaneous injection of 0,10 g of caffeine

us to demonstrate our findings in every case on two figures, as the radiographs taken 1/2 hour (1/2 h. p. c.) and 4 hours (4 h. p. c.) subsequent to ingestion of the contrast medium offer a characteristic illustration of the behaviour of the whole gastro-intestinal tract. As contrast medium we used *Notobarium* of which 10 g per kg was introduced by means of a human duodenal tube. The radiographs marked »a« represent conditions 1/2 h. p. c., those marked »b« 4 h. p. c.

»1/2 h. p. c. after subcutaneous injection of 0,10 g of caffeine: the half evacuated stomach shows normal tone, animated peristalsis; the rest of the contrast paste is visible in the small intestines and has not yet reached the coecum. The lumen of the small intestines is normal, the stomach is empty, the smaller part of the paste is discernible in the small intestines while the bulk is

gradually filling up the large intestine, but has not yet reached the ampullae. (See 1.b.)

1/2 h. p. c. following ingestion of black coffee (containing about 0,20 g caffeine) : Slightly more than half the paste still in the stomach, the rest in the mall intestine and has not yet reached the coecum. The tone of the stomach is good with lively peristalsis. The peristalsis of the jejunal loops is increased (See 2.a). 4 h. p. c. : The stomach is empty, some of the paste is in the small intestine, the greater part is in the large intestine filling it gradually up. The

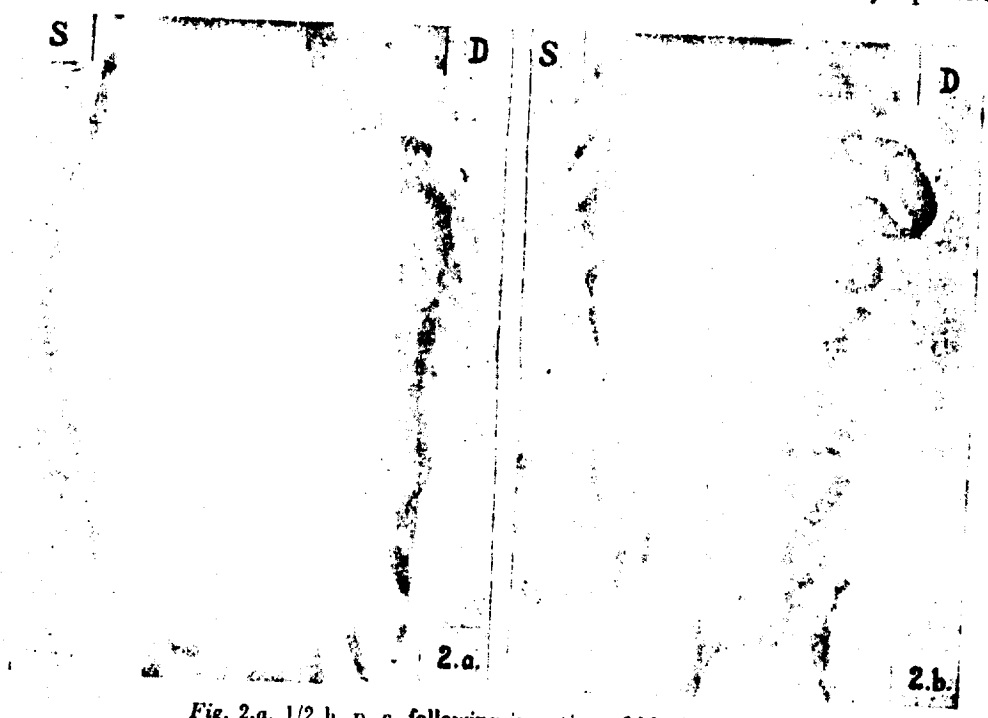

Fig. 2.a. 1/2 h. p. c. following ingestion of black coffee
Fig. 2.b. 4 h. p. c. following ingestion of black coffee

ampullae have not yet been reached, the deep plicae of the large intestine display a spastic character (See 2.b).

1/2 h. p. c. after 0,25 g caffeine administered intravenously : About half of the contrast medium is in the stomach, the rest in the small intestine, the coecum has not been reached. The front jejunal loops of the small intestine show heightened peristalsis and tone (See 3. a). 4. p. h. c. : The stomach is empty, part of the ileal loops of the small intestine and the large intestine are evenly filled up, displaying increased tone (See 3. b). Passage from the small intestine into the large intestine, marked with an arrow, is clearly discernible.

1/2 h. p. c. after intravenous injection of 0,50 g caffeine : One quarter residue in the stomach, the rest of the paste is in the small intestine without

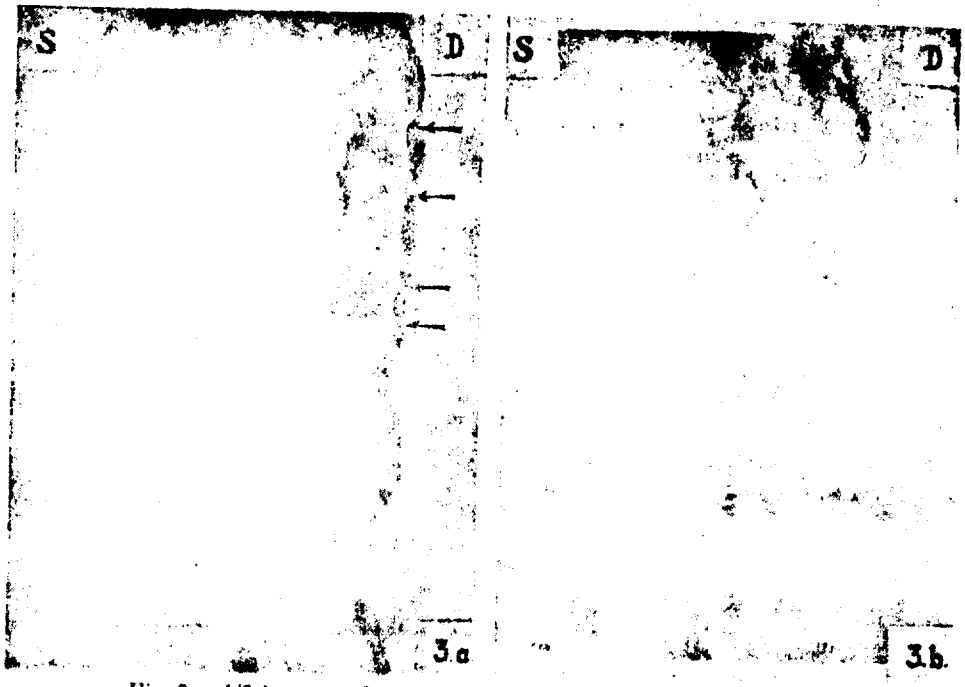

Fig. 3.*a*. 1/2 h. p. c. after intravenous injection of 0,25 g caffeine
Fig. 3.*b*. 4 h. p. c. after intravenous injection of 0,25 g caffeine

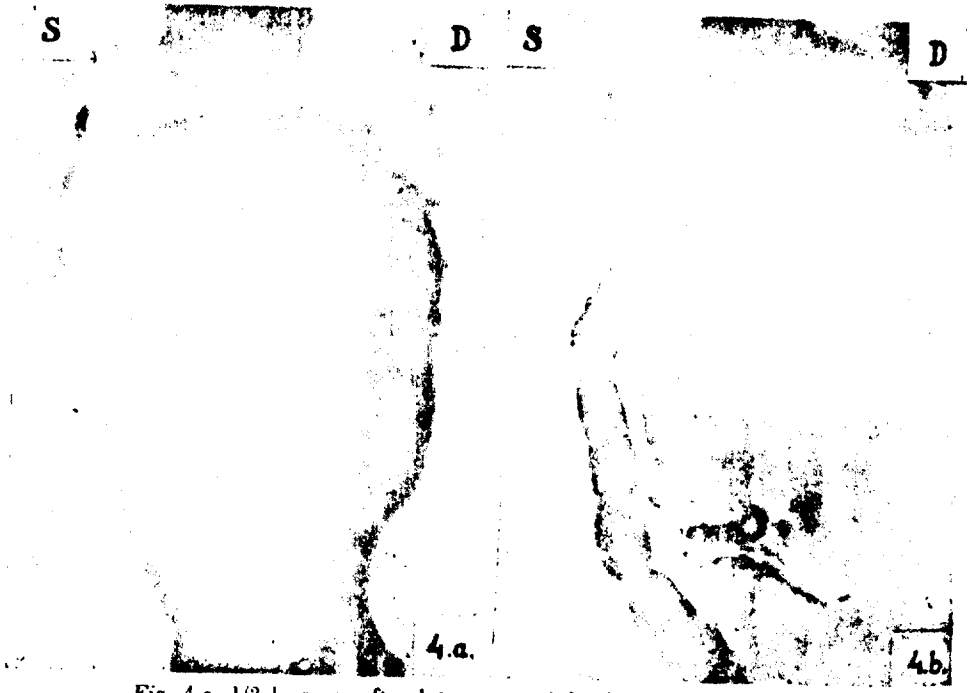

Fig. 4.*a*. 1/2 h. p. c. after intravenous injection of 0,50 g caffeine
Fig. 4.*b*. 4 h. p. c. after intravenous injection of 0,50 g caffeine

having reached the coecum (See 4. a). On account of accelerated evacuation, the radiograph taken 1/2 h. p. c. merely suggests good tone of the stomach, while the animated peristaltic waves are visible on the picture. Especially remarkable is the complete relaxation of the pylorus, causing the antrum pilory to disappear. The tone of the small intestines is lowered, their lumen perceptibly dilated with even but slackened peristalsis. 4 h. p. c. : The stomach is empty, some sporadic residue is visible in the small intestine, the rest of the paste is in the large intestine, and has reached the ampullae. The tone of the colon is normal (See 4.b).

Conclusions

The evaluation of the action of caffeine on gastro-intestinal peristalsis and tone calls for comparison of our relative observations to the results we published on our findings obtained from drug-free subjects. The complete evacuation of the stomach was found to average 1 hour 26 minutes. The paste took an average of 1 hour and 57 minutes to appear in the coecum, 4 hours and 45 minutes to reach the ampullae. If we accept these average figures as the basis for an estimate, the passage values of our caffeine tests may be determined as follows : the evacuation of the stomach is accelerated in every case. This acceleration is the most remarkable after the intravenous injection of 0,5 g caffeine, when no more than one quarter remnant of the paste can be traced in the stomach 1/2 h. p. c. With the other three forms of dosage the 1/2 h. p. c. residue left in the stomach amounted to 1/2 of the ingested contrast medium, which means half-time evacuation compared to normal conditions. On 1 h. p. c. radiographs not reproduced here, we have in every case found the stomach empty, which corresponds to a faster rate of evacuation by 26 minutes compared to tests performed on untreated laboratory animals. Different kinds of dosage do not appear to have evoked any essential change in gastric tone, hence increased evacuation may be safely attributed to heightened peristaltic frequency ; the process can be easily followed up by radioscopy.

As mentioned above, the time required for the paste to reach the coecum has served as a basis for assessing the motility of the small intestines. Under normal conditions it takes somewhat less than 2 hours. Only figure 4.a demonstrates a relative deviation, the posterior ileal loops being also full 1/2 h. p. c., but the paste has not yet reached the coecum. This radiograph was taken of the 0,50 g caffeine dose injected intravenously, when gastric evacuation was the most rapid. The moderately increased motility of the small intestine may be conceived of as the proportionate consequence of increased gastric evacuation. Animated peristalsis has been observed in the jejunal loops only at the front section, displaying brisk undulation. The deepest waves appear subsequent to the intravenous administration of 0,25 g of caffeine (See Fig. 3, marked with arrows) ; the shallowest are seen on the radiographs taken after the intrave-

nous injection of 0,50 g caffeine (See Fig. 4). It seems probable that, up to a certain amount (in the present case 0,25 g intravenously), caffeine stimulates the tone and deepens the contractions of the small intestines, while the effect of larger doses manifests itself in diminishing the tone of the small intestines.

The third phase of our passage examinations dealing with the function of the large intestines, is illustrated by the figures marked »b«. In our tests performed on untreated subjects we found that the progress of the paste takes an average of four hours 45 minutes to reach the ampullae. Among our radiographs only 4. b reveals the appearance of paste in the ampullae, i. e. the case where the intravenous injection of 0,50 g caffeine is attended by increased motility of the stomach and the small intestine. All the other radiographs show the appearance of the paste in the descending colon. The tone of the colon is a faithful mirror of passage conditions. According to Fig. 4. b, the tone of the colon is normal, whereas radiographs No. 1, 2, 3. b show the spastic character of the colon.

The degree of evacuation was eventually decided by the behaviour of the colon, in as much as the spastic condition of the intestines and the diminished motility counterbalanced the increased evacuation of the stomach and the animated peristalsis of the anterior jejunal loops.

We should like to complete our report by adding that in the examinations performed 12 h. p. c. the intestines were invariably found to be evacuated, which tallies with normal conditions of 8—12 hours' excretion time.

SUMMARY

1. The effect of caffeine on the gastro-intestinal tract of dogs has been found to vary, depending on the amount and mode of dosage.

2. The effect of an oral black coffee dose containing approximately 0,20 g of caffeine is roughly similar to the action obtained by 0,10 g caffeine injected subcutaneously.

3. Each experiment showed increased gastric evacuation, particularly after intravenous injection of 0,50 g of caffeine.

4. Every mode of dosage provokes animated peristalsis in the initial sector of the small intestines. Intestinal tone is at the lowest after the i. v. administration of 0,50 g of caffeine, while intestinal contraction would appear to be the most marked subsequent to the intravenous injection of 0,25 g.

5. Except following the intravenous injection of 0,50 g of caffeine, the large intestines display signs hinting at increased tone and spastic contraction; accordingly the passage time of the large intestines is moderately protracted.

6. The evacuation time of the paste on the whole corresponds to normal conditions.

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РЭНТГЕНОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ ПРОХОЖДЕНИЯ СОДЕРЖИМОГО ЖЕЛУДОЧНО-КИШЕЧНОГО ТРАКТА ПОСЛЕ ВВЕДЕНИЯ КОФЕИНА

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Изучая действие кофеина на быстроту прохождения содержимого желудочно-кишечного тракта, авторы установили:

1. что в зависимости от дозы и способа аппликации получается разный эффект кофеина на желудочно-кишечный тракт собаки;
2. что фармакодинамический эффект количества натурального кофе, т. н. »двойное черное« при выпивании, равнялся эффекту 0,1 г кофеина, введенного подкожно;
3. что кофеин в дозе 0,5 г при внутривенной аппликации усиливает скорость прохождения содержимого через желудок;
4. что каждое введение кофеина усиливает перистальтическое движение начального участка тонкого отдела кишечника, при чем самый слабый тонус наблюдается при внутривенном введении 0,5 г кофеина, тогда как очень выраженная контракция кишек наблюдается при внутривенном введении 0,25 кофеина;
5. что толстый отдел кишечника, за исключением введения внутривенно 0,5 кофеина, показывает признаки повышенного тонуса и спастических контракций, благодаря чему скорость прохождения содержимого умеренно замедляется;
6. что прохождение кашицы в целом не отличается от нормы.

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TERATOGENICITY OF CAFFEINE IN MICE RELATED TO ITS MODE OF ADMINISTRATION

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In teratological experiments with drugs, the injection by ways of parenteral routes is often used for the advantage of securing certain effects, especially exact dosage-response relation. It has been presumed that the teratogenic action of a drug varies with the mode of parenteral administration, even if the same total daily dosage is employed and this may be due to the variations in the nature of metabolites of a drug and their concentration or duration in the embryonic tissues. There is little information in this area at hand. Isaacson and Chaudhry (1) reported that the incidence of cortisone-induced cleft palate in mouse embryos is higher when administration of 4 small doses at 6-hour interval is given rather than a single dose of the total amount.

The teratogenicity of caffeine in mice was established and some of its mechanisms were studied by our group (2-4). The rapid metabolic rate and no apparent accumulation in the body of this compound established in man (5) can be presumably applied to other mammals. So the administration of caffeine could be an advantageous tool for analytical studies of teratogenicity in experimental mammals. This study was undertaken to establish a relationship between teratogenic activity and some modes of administration by using this compound.

METHODS

The ICR-JCL mice at the weaning age supplied by the Central Laboratory for Experimental Animals (Meguro, Tokyo) were raised for 6 weeks at our Laboratory (Okazaki). During this period as well as throughout the experiments mentioned below, they were kept in an air-conditioned room at 22°±1°C and 55±5% of humidity and given the compressed pellets for mice and rats (OA-2) manufactured by Japan CLEA Co. Ltd. (Meguro, Tokyo) and fresh tap water *ad libitum*.

Nulliparous females of 9-13 weeks of age were mated all night (17 hours) with adult males. Copulation was checked by the presence of a vaginal plug and the day when the plug was found, was designated as day zero of gestation. One hundred and sixty-five pregnant mice were divided into eight groups of about equal numbers on day 12 of gestation and they were treated as follows:

Group Ia: 1% aqueous solution of caffeine (J.P.) was injected once at 9:30 a.m.

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at a dosage of 200 mg/kg into the nape subcutaneously.

Group IIa: The same solution was injected once at 9:30 a.m. [illegible] intraperitoneally.

Group IIIa: The same solution was injected twice at 9:30 a.m. and 11:30 a.m. [illegible]

TABLE 1. Effect of prenatal administration of caffeine (1% aqueous solution) given on gestational day 12 upon mouse offspring observed at term.

Group	No. of mothers treated	Dosage at single injection (mg/kg)	Total implants (per litter)	Dead or resorbed (%)	No. of mothers with abnormal fetuses (%)	Live fetuses		
						No. of live fetuses (per litter)	Average body weight ±Sx(g)	No. of abnormal (% out of total)
Ia	20	200	287 (14.4)	39* (13.6)	14* (70.0)	248 (12.4)	1.28* 0.06	51* (20.6)
IIa	20	200	273 (13.7)	22+ (8.1)	14 (70.0)	251 (12.6)	1.27* 0.007	46++++ (18.3)
IIIa	23	100	308 (13.4)	43++ (14.0)	11* (47.8)	265 (11.5)	1.32* 0.007+++	18* (6.8)
IVa	22	100	307 (14.0)	45+++ (14.7)	11* (50.0)	262 (11.9)	1.27* 0.006	16* (6.1)
Ib	21	0	262 (12.5)	18 (6.9)	1 (4.8)	244 (11.6)	1.36 0.007	1 (0.4)
IIb	19	0	235 (12.4)	17 (7.2)	3 (15.8)	218 (11.5)	1.36 0.008	3 (1.4)
IIIb	20	0	261 (13.1)	23 (8.8)	1 (5.0)	238 (11.9)	1.36 0.006	1 (0.4)
IVb	20	0	268 (13.4)	18 (6.7)	1 (5.0)	250 (12.5)	1.33 0.007	1 (0.4)

Ia : administered once subcutaneously ; IIa : administered once intraperitoneally ; IIIa : administered twice intraperitoneally at an interval of 2 hours ; IVa : administered twice intraperitoneally at an interval of 4 hours ; Ib, IIb, IIIb and IVb : respective control groups for Ia, IIa, IIIa and IVa.

* : significantly different from those of respective control groups ($P<0.01$)

+ : significantly different from that of group Ia ($P<0.05$)

++ : significantly different from that of group IIa ($P<0.05$ respectively)

+++ : significantly different from that of groups IIa and IVa ($P<0.01$)

++++ : significantly different from that of groups IIIa and IVa ($P<0.01$)

2-hour interval) at a dosage of 100 mg/kg respectively intraperitoneally.

Group IVa: The same solution was injected twice at 9:30 a.m. and 1:30 p.m. (at 4-hour interval) at a dosage of 100 mg/kg respectively intraperitoneally.

Group Ib, IIb, IIIb and IVb: The same volume of saline was injected in the same way with the above mentioned groups as the respective control groups.

All the mothers were sacrificed on day 18 of gestation. The implantation sites and maceration of the fetuses were inspected *in situ* and all living fetuses were removed, weighed and examined for external deformities including those in the oral cavity.

RESULTS AND DISCUSSION

Most animals of group Ia and IIa showed transient dyspnea and slight convulsions several minutes after the caffeine injection, while those of group IIIa and IVa exhibited transient dyspnea and depressed activity after the injection. All the mothers survived until term.

The main effects on the offspring observed on day 18 of gestation are shown in Table 1.

The incidence of dead or resorbed fetuses in the caffeine treated groups is higher than that of each of the respective control group. Number of the mothers with the abnormal fetuses in the caffeine treated groups is more than that of respective control group. It was also shown that the incidence of the fetuses with abnormalities in each of the caffeine treated groups is significantly higher than that of the respective control groups.

If the group Ia is compared with the group IIa, lethal effect is severer in the group

TABLE 2. Classification of abnormal fetuses according to type of defects.

Group	No. of live fetuses	No. of fetuses with abnormalities	Incidence of fetuses with each abnormality (% out of live)				
			Cleft palate	Micrognathia	Defect of digits	Hematoma	Clubfoot
Ia	248	51	48* (19.4)	15* (6.0)	1 (0.4)	15* (6.0)	3 (1.2)
IIa	251	46	41*+ (16.3)	11*+ (4.4)	–	11*++ (4.4)	3 (1.2)
IIIa	265	18	16* (6.0)	1 (0.4)	1 (0.4)	5* (1.9)	2 (7.5)
IVa	262	16	5 (1.9)	–	–	–	11* (4.2)
Ib	244	1	–	–	–	–	1 (0.4)
IIb	218	3	–	–	–	–	1 (1.4)
IIIb	238	1	1 (0.4)	–	–	–	–
IVb	250	1	1 (0.4)	–	–	–	–

* Significantly different from those of respective control groups ($P<0.01$)
\+ Significantly different from those of group IIIa and IVa ($P<0.01$)
++ Significantly different from that of group IVa ($P<0.01$)

Ia than in the group IIa. However, no significant difference of the incidence of mothers with abnormal fetuses as well as that of the malformed fetuses is shown between the group Ia and IIa.

Next, the comparison among the three intraperitoneally injected groups, IIa, IIIa and IVa, revealed the findings that the lethality is higher in the groups IIIa and IVa than in the group IIa, while the incidence of the fetuses with abnormalities higher in the group IIa than in the groups IIIa and IVa. Also it was found that there is no appreciable difference of the effects between the group IIIa and IVa.

The fetuses with abnormalities are classified according to types of defects in Table 2.

All treated groups showed the high incidence of cleft palate and the occasional occurrence of clubfoot. The hematoma was accompanied in most cases by some adjacent skeletal defects. The incidence of cleft palate in the group IIa is significantly higher than that of the groups IIIa and IVa. The same tendency is shown in the incidence of micrognathia. Subcutaneous hematoma was found around the mouth and at the digits in the three treated groups.

The above mentioned findings indicate some significant facts on the dependence of embryotoxic effects of caffeine upon the mode of its administration. That is, the subcutaneous injection of its single dose induces a severer lethal effect on the embryos than the intraperitoneal injection of the same dose, but no difference in the teratogenic effect is shown between these two routes; by the intraperitoneal route, administration of two divided small doses at 2- or 4-hour intervals produces severer lethality, and lower teratogenicity than that of the single total dose. There is no doubt that the rate of absorption following subcutaneous injection of caffeine is lower than intraperitoneal injection. It may be possible that administration of a single dose leads to the higher concentration and the shorter duration of caffeine or its metabolic products in the blood as well as the embryonic tissues than administration of the divided small two doses at the interval. Although with what drug metabolism in mothers and embryos such variations in embryotoxicity are linked awaits further studies, the above results may be accounted for by the speculation that the lethality of caffeine on the mouse embryos is related to the duration of exposure of the embryos to the compound or its metabolic by-products while the teratogenicity is rather attributable to the sufficiently high concentration. At any rate, dissociation of both lethality and teratogenicity to the embryos revealed by varying the mode of administration might suggest some difference in the pathway involved in causing both effects.

SUMMARY

Caffeine was administered to the ICR-JCL pregnant mice on day 12 of gestation at the dosage of 200 mg/kg causing transient maternal toxic symptom by four different methods: once subcutaneously, once intraperitoneally, twice at 2-hour interval at the half dosage intraperitoneally and twice at 4-hour interval at the half dosage intraperitoneally. The fetuses were examined at term externally. Main findings are as follows:

1. Lethal effect of a single subcutaneous injection is severer than that of a single intraperitoneal injection.

2. A single intraperitoneal injection causes higher teratogenic effects as well as causation of subcutaneous hematoma in the jaws and digits, but lower lethality than the two times administration of half dose at 2- or 4-hour interval. Such differences in teratogenic effects are conspicuous in the facial defects such as cleft palate and micrognathia.

Acknowledgement: We greatly acknowledge the helpful advice of Dr. Toshio Watanabe, Director of the Research Laboratory, Nippon Merck-Banyu Co. Ltd.

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Adverse Effects of Prolonged Administration of Caffeine on Rat Fetus

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Adverse Effects of Prolonged Administration of Caffeine on Rat Fetus. FUJII, T., AND NISHIMURA, H. (1972). *Toxicol. Appl. Pharmacol.* 22, 449-457. Caffeine was mixed in a diet at 0.25 or 0.50% by weight and given to rats throughout or during each of selected periods of gestation. Near term fetuses were examined for external, visceral and skeletal abnormalities. Fetal blood obtained from the umbilical vein was examined for nucleated red blood cells. Maternal blood obtained from the abdominal aorta was also examined for cholesterol concentration. The major findings were as follows: (1) the effects of caffeine on the fetus appeared dependent on the time of administration during gestation; (2) a high incidence of fetal resorption and occurrence of certain types of gross, visceral and skeletal abnormalities of live fetuses were found when the compound was given throughout or during the middle stage of pregnancy; (3) generalized edema was present in the majority of fetuses when caffeine was given throughout or during the late stage of pregnancy. The severity and frequency of edema were clearly dose dependent; (4) ossification was delayed in all the treatment groups, and the delay was more severe in the fetuses with edematous change; (5) increase of normoblasts in the fetal blood was found in the group where fetal edema was manifested; and (6) the average cholesterol concentration in the maternal blood serum measured near term was higher in the animals treated throughout pregnancy than in the controls, although the difference was not significant.

It has been reported that an excessive dose of caffeine produces malformations in mice (Nishimura and Nakai, 1960; Nakai, 1961; Knoche and König, 1964; Fujii *et al.*, 1969; Fujii and Nishimura, 1969), rats (Bertrand *et al.*, 1965; Georges and Denef, 1968; Groupe d'étude des Risques Tératogènes, 1969; Fujii, unpublished data) and rabbits (Groupe d'étude des Risques Tératogènes, 1969) when the compound is administered to females during the period of major organogenesis. Pitel and Lerman (1964) reported that caffeine causes opacification of the fetal rat lens when injected directly into fetuses on day 17 of gestation. It was confirmed that caffeine passes readily through the placenta into the mouse embryo (Tanimura and Terada, 1969) or penetrates the preimplantation blastocyst of the rabbit (Fabro and Sieber, 1969).

In the present experiment, we studied the effects of prolonged administration of caffeine on developing rat fetuses by giving females a diet containing caffeine throughout or during selected periods of gestation. The use of dietary administration during these

periods may be justified by the fact that caffeine is quickly absorbed from the gastrointestinal tract in the rabbit (Sant'Ambrogio *et al.* 1964).

METHODS

Sprague-Dawley rats purchased from Japan CLEA Co. at 4 wk of age were conditioned to our laboratory environment until 10 wk of age. Then, each female was mated overnight (17 hr) with an adult male of the same strain. Copulation was established by the presence of spermatozoa in the vaginal smear. On this day, designated as day 0, animals considered to be pregnant were assigned to each of 7 groups described below. Caffeine was mixed in commercial powdered rat diet (CA-1, Japan CLEA Co.) at 0.25 or 0.50% by weight. Five of the groups were fed this diet ad libitum during selected periods and the basal powdered diet during other periods of gestation. Two other untreated groups served as controls for the caffeine treated groups. Fresh drinking water was available throughout the experiments.

Group I: fed the diet containing 0.25% caffeine throughout gestation.

Group II: fed the diet containing 0.50% caffeine throughout gestation.

Group III: fed the basal powdered rat diet throughout gestation. This group served as the control for groups I and II. Pregnancy of animals in groups I, II and III was terminated on day 21 of gestation.

Group IV: fed the diet containing 0.50% caffeine from day 1 to 7 of gestation.

Group V: fed the same caffeine containing diet from day 8 to 14 of gestation.

Group VI: fed the same caffeine containing diet from day 15 to 21 of gestation.

Group VII: fed the basal powdered diet throughout gestation. This group served as the control for the groups IV, V and VI. Pregnancy of the animals in the groups IV, V, VI and VII was terminated on day 22 of gestation.

Food consumption and body weight of the animals in groups I, II and III were measured daily throughout pregnancy. About 10 ml of blood was obtained from the abdominal aorta just prior to sacrifice. Total serum cholesterol was determined by a modified Zak-Henly method (Niwa *et al.*, 1968). A small amount of fetal umbilical blood was taken immediately after removal of the placenta from a few of the littermates of the females in groups IV, V, VI and VII and was used for blood counts and preparation of blood smears stained with Giemsa solution.

After sacrifice, the uterus was opened and examined for implantation sites, resorptions and dead fetuses. Live fetuses were removed from the uterus, weighed and examined for external malformations. They were then fixed either in Bouin fluid for examination for internal malformations by the method of Wilson (1965), or in 95% alcohol for detection of skeletal defects after preparation of cleared specimens stained with alizarin red S (Dawson, 1926). Several placentas from groups I, II and III were fixed in 10% formalin solution for histologic examination.

RESULTS

Groups I, II and III

None of the females showed noticeable behavioral change during caffeine administration. Maternal weight gain of group I was similar to that of the control group. In

group II, the females gained significantly less weight than the control group throughout pregnancy. Food consumption was less in both treated groups I and II during the first week, but showed recovery thereafter. Average daily drug intake during pregnancy was about 180 mg/kg in group I and 330 mg/kg in group II.

The effects of caffeine on development of the fetuses observed near term are shown in Table 1. In both treated groups, embryotoxic effects, such as increase of fetal resorption and decrease of body weight of live fetuses, were observed. A dose effect relationship was clearly shown with these parameters.

FIG. 1. Edematous fetuses from different litters of the group where a diet containing 0.50% caffeine was given to females throughout pregnancy.

The most striking change in fetuses in both treatment groups was generalized edema. Especially in group II, all live fetuses showed severe edematous change (Fig. 1) which in a few was accompanied by sc hemorrhage.

The results of visceral and skeletal examination are summarized in Table 2. In group II, the frequency of malformed fetuses as well as various types of malformations or lesions, such as hydrocephaly, intravitreous hemorrhage, misplaced esophagus and ventricular septal defect was significantly higher than in the control group. Ossification was severely delayed in both treatment groups. In group II, most of the placentas examined showed marked congestion of maternal capillaries.

Although the total cholesterol concentration in the maternal sera was slightly higher in the treated groups, the difference between either of both treatment groups and the control group was not significant. Furthermore, no dose dependent change was shown.

TABLE 2

INTERNAL AND SKELETAL OBSERVATION OF RAT FETUSES WHOSE MOTHERS WERE FED A DIET CONTAINING 0.25 OR 0.50% CAFFEINE THROUGHOUT PREGNANCY

	Internal observation						Skeletal observation				
			Malformations					Hypoplasia			
Group	No. examined	Total malformed	Hydrocephaly	Intravitreous hemorrhage	Esophagectopy	Ventricular septal defect	No. examined	Supraoccipital	Sacral vertebra	Average no ossification center caudal v body SI	No. malformed
I (0.25%)	62	[illegible]	1 (1.6)	0	2 (3.2)	0	66	[illegible] (4.5)	2 (3.0)	[illegible] 0.11	0
II (0.50%)	37	21[a] (56.8)	5[b] (13.5)	10[b] (27.0)	7[b] (18.9)	3[c] (8.1)	37	28[b] (75.7)	27[b] (73.0)	0.51[b] 0.17	1[d]
III (Control)	83	[illegible]	0	0	0	0	90	0	0	4.46 0.07	0

[a] Parentheses (a) % [illegible] ned.
[b] Significant at P 0.01
[c] Significant at P 0.05
[d] Wavy ribs.

Groups IV, V, VI and VII

Table 3 summarizes the major findings of the experiments where the caffeine containing diet at 0.50% was fed during selected periods of pregnancy. A significantly high rate of resorption was observed in group V. Fetal weight was significantly suppressed in

TABLE 3

EFFECT OF 0.50% CAFFEINE IN THE DIET ON THE DEVELOPMENT OF THE RAT FETUS WHEN GIVEN DURING VARIOUS PERIODS OF GESTATION

Group (treated period)	No. mothers	Total implants	No. resorptions (%)	Average litter size	Average body weight SE (g)	No. malformed	No. edema
					Live fetuses ( ): % out of total live		
IV (1–7)	16	217	24 (11.1)	12.9	4.88[b] 0.036	2 (1.0)	0
V (8–14)	15	201[a]	56[b] (27.9)	10.4	4.33[b] 0.040	20[b] (13.8)	1 (0.7)
VI (15–21)	13	176	11 (6.3)	12.7	5.23 0.050	0	158[b] (95.8)
VII (Control)	13	180	10 (5.6)	13.1	5.25 0.031	0	0

[a] Exclude the number from a case of whole litter resorption.
[b] Significant at $P < 0.01$.

FIG. 2. Edematous change of a fetus (right) from a female fed a diet containing 0.50% caffeine from day 15 to 21 of gestation. Compare with a normal control on the left.

groups IV and V. A significantly high frequency of external malformations, all tail defects such as short or kinky tail, was found in group V. Occurrence of edema (Fig. 2) in live fetuses was essentially confined to group VI, and its frequency was as high as 95.8%.

Visceral malformations are presented in Table 4. A high incidence of hydronephrosis was found only in group IV, whereas a high incidence of an esophageal defect was present in group V. Skeletal examination of the fetuses revealed that ossification was retarded in all the treatment groups, and the grade was most severe in group VI.

TABLE 4

VISCERAL MALFORMATIONS CAUSED BY 0.50% CAFFEINE FED IN THE DIET DURING VARIOUS PERIODS OF GESTATION

Group (treated period)	No. examined	No. malformed	Hydro-cephaly	Esopha-gectopy	Ectopic heart	Hydro-nephrosis	Absence of kidney
IV (1–7)	101	10[b] (10.0)[c]	0	0	0	9[a] (8.9)	1 (1.0)
V (8–14)	77	11[a] (14.3)	1 (1.3)	10[a] (13.0)	1 (1.3)	0	0
VI (15–21)	84	1 (1.2)	0	0	0	1 (1.2)	0
VII (Control)	84	1 (1.2)	1 (1.2)	0	0	0	0

[a] Significant at $P < 0.01$.
[b] Significant at $P < 0.05$.
[c] Value in parentheses shows percentage.

The results of blood counts and examination of the smear of fetal blood are listed in Table 5. From these results, it can be concluded that the number of nucleated red blood cells was significantly increased in group VI.

TABLE 5

AVERAGE VALUE OF BLOOD CELL COUNTS ON RAT FETUSES WHOSE MOTHERS WERE FED A DIET CONTAINING 0.50% CAFFEINE DURING VARIOUS PERIODS OF GESTATION

Group (day of gestation)	RBC ($\times 10{,}000/mm^3$)	Nuc. RBC[a] ± SE (per mm^3)	Nuc. RBC/ 100 WBC[c]
IV (1–7)	210 $n = 28$	248 ± 25.8 $n = 35$	415 $n = 32$
V (8–14)	188 $n = 27$	244 ± 21.8 $n = 27$	541 $n = 28$
VI (15–21)	187 $n = 14$	571 ± 43.1[b] $n = 28$	770 $n = 30$
VII (Control)	201 $n = 17$	192 ± 13.5 $n = 26$	385 $n = 27$

[a] 1/50 value.
[b] Significant at $P < 0.01$.
[c] Calculated from differential leukocyte count.

DISCUSSION

A dose effect relationship was apparent with respect to embryotoxicity, i.e., lethality, growth suppression and edema, when the pregnant females were given 0.25 or 0.50%, caffeine in the diet throughout pregnancy. Toxic effects on the fetuses in group II appeared more severe than those observed in groups IV, V and VI, and in addition, intravitreous hemorrhage was found only in females from group II. Comparing the toxic changes and abnormalities found in fetuses of groups IV, V and VI, there is a certain difference among them. Lethal and teratogenic effects were most pronounced in the group given caffeine from day 8 to 14, but characteristic edema was found only in the group treated from day 15 to 21.

In another experiment, in which the same treatment as in group V was given to a few female rats, pregnancy was terminated on day 15 just after the treatment was finished. The embryos were examined for edematous changes to see whether the edema disappeared during the following untreated period. Edema was not observed in these embryos (Fujii, unpublished data). When a single ip injection of 175 mg/kg of caffeine was given to Sprague-Dawley rats on day 14 of gestation, malformations found in their fetuses included cleft palate, micrognathia, glossopalatine fusion, digital defects and sc hematoma, but no edema (Fujii, unpublished data). Comparing the results in the groups I, II and VI and considering the above results, it is suggested that the continuous administration of caffeine during some late period of pregnancy causes this edematous change in the fetus.

Fischer (1940) found fetal edema associated with growth retardation, congestion of the placenta, sc hemorrhage and placental necrosis, when 100–200 mg/kg of caffeine was administered po to female rabbits daily from the stage prior to conception through the gestational period until day 18 or 20, when pregnancy was terminated. The effects in the fetuses were similar to ours, except that placental necrosis was not seen in our study. With respect to fetal edema, Miyamoto (1969) has reported that frequent and severe edema of the fetus was caused by po administration of aspirin or sodium salicylate at 600–660 mg/kg/day during day 14 to 17 of gestation in the rat. Posner and Darr (1970) reported that fetal edema as well as oral-facial malformations occurred after the administration of 4-benzhydrylpiperazines to rats from day 13 to 16 of gestation. Horger and Hutchinson (1970) reported that hydrops in the fetal lamb was induced when cycloheximide, an inhibitor of protein synthesis, together with phenylhydrazine was injected into the fetus at a late stage of gestation. In these studies, too, it appears that fetal edema can be caused in experimental animals by prolonged administration of a certain compound during the late period of gestation.

That edema was accompanied by an increase in normoblasts suggests some association between both findings. Such experimental results may have a certain relation to the known fact that erythroblastosis fetalis is occasionally accompanied by hydrops fetalis.

Naismith *et al.* (1969) measured serum cholesterol of rats fed coffee or caffeine for 54 days and found that plasma cholesterol concentrations rose with increasing intake of caffeine. Also, cholesterol has been reported to be teratogenic, producing palatal abnormalities in the rat (Buresh and Urban, 1964). However, our investigation did not show a definite increase of serum cholesterol in the groups treated throughout gestation although average values were higher than those of the controls.

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INTRODUCTION

An attempt to enhance running performance of race horses by means of caffeine-containing drinks such as coffee, coka cola, etc., or caffeine agents has long been practised (6), and studies have been conducted on this subject (7). In the doping of race horses, caffeine is one of the materials which bring about the highest positive effect. However, few investigators, other than MacKay (4), have conducted an experiment on the relationship between caffeine and running performance of horses. The authors studied the effects of caffeine on race horses as a sequel to the study on trans-π-oxocamphor (1), the results of which are reported below.

EXPERIMENTAL MATERIALS AND PROCEDURES

EXPERIMENTAL HORSES: Three thoroughbreds (Sen; age, 6; weight, approximately 420-500 kg) owned by the Abalon Riding School were used. As a preliminary experiment, caffeine was administered to the horses while they were resting during a period from January 24, 1968 to May 7 (temperature, 7.0 - 18.0°C; humidity, 39 - 88%). Subsequently, running tests were carried out between May 15 and June 19 (temperature, 17.5 - 26.0; humidity, 36 - 73%).

DRUGS: An official 20% sodium benzoate caffeine injection solution, a most commonly used caffeine agent, was employed. The doses were 1.3, 2.5, 3.8, and 5.0 g per horse (normal dose, 3 g) for the preliminary experiment, and 2.5 and 5.0 g for the running experiment. The drug was injected by the hypodermic route. In both types of experiment, the control received 25 ml of physiological saline solution which is equal in volume to 5.0 g of sodium benzoate-caffeine.

In the preliminary test, the caffeine was given at 2 weeks intervals with the dose gradually increased from the lowest. In the running experiment, the compound was given once a week at a dose randomly selected at each test.

RUNNING TEST: The tests were conducted as described in the previous paper (Figure 1). The standard lap time (the standard velocity in the previous paper) was changed to 50 sec/round of regular gait (114 m/min), 25 sec/round (228 m/min) of trot, 17 sec/round (335 m/min) of canter. A control experiment at a standard trot rate of 13 sec/round (438 m/min) was included for No. 3 horse only. The exercise was carried out 1 hour after the injection of the drug, until which time the horses were stabled.

OBJECT OF OBSERVATION: In the preliminary experiment, the reins were moderately drawn for considerable lengths in the stable, thereby moderately restraining the horse by the head only. The ECG was continuously recorded by A-B lead from 30 min before the injection to 3 hours later by the wireless remote measurement method. The respiration rate was visually examined at 10 min intervals, and the body temperature was taken once before injection and at 30 min intervals after injection. The behavioral changes were also recorded.

In the running tests, the procedure described in the previous paper (1) was followed, including ECG recording, lap time measurement, and riders' subjective evaluation. Also added to the procedure was the measurement of the number of gaits (complete gaits) per round and the respiration rate while standing prior to the test, immediately after the end of the test, and 3, 5, 10, 15, 20, and 30 min after recovery.

EXPERIMENTAL RESULTS

1. PRELIMINARY EXPERIMENT

No significant change was produced by the drug except for slight changes in heart beat and behavior at over 3.8 g. The control indicated a gradual decrease

in heart beat or a periodic change consisting of a decrease, a recovery to the preexperimental level, then a drop. At over 3.8 g, such changes diminished, and, in many cases, the preinjection values were maintained (Figure 2). The control exhibited a state of drowsiness intermittently, comprising the drooping of the head, laying of the weight of the head and neck on the reins while standing, etc. However, 3.8 g or more of caffeine resulted in the complete diminution of these drowsy signs within 15 minutes at the earliest and 1 hour at the latest. The sodium benzoate caffeine did not produce excessive stimulation, but one case (No. 2) exhibited signs of severe hyperkinesia for a period of 6 - 13 min after injection at 3.8 g, and remained hypersensitive to sounds for 3 hours, with occasional loud neighing possibly due to psychological excitation. In a follow-up test 1 month later, the horse only indicated diminution of the drowsy signs, with no indication of hyperkinesia or psychological excitation. Even at 5.0 g, no change other than loss of drowsy signs was noted.

On the basis of these results, a dose of 2.5 g was regarded as producing no change in a horse at rest and a dose of 5.0 g as producing positive changes. These doses were used for the running tests. The horses were tested 1 hour after injection.

2. RUNNING TESTS

LAP TIME: The lap time became shorter due to the enhanced forwarding capacity of the horse stimulated by the caffeine (Figure 3) despite the rider's effort to maintain the standard lap time set in advance. The reduction of lap time occurred even at 2.5 g at which no change was observed in the preliminary experiment. For instance, horse No. 1 showed a change from 4 to 9 rounds and horse No. 2, a change from 1 to 5 rounds. Horse No. 3 increased its velocity to such an excessive degree that it went over the track, and reentered the track after readjusting its posture. At 5.0 g, No. 2 did not fully perform in a round

small-diameter track. According to the rider, an enhancement in the impulse to advance was noted in any mode of running, and, with increasing intensity of exercise, the control of the lap time within the standard range became difficult and the performance of the horse gained momentum.

ECG WAVE PATTERN: At 2.5 g, No. 2 indicated a series of arrythmic signs comprising the 2nd atrioventricular heart blocks successively after the completion of exercise followed by sinoauricular blocks 3 times (Figure 4). There was no other abnormality.

HEART BEAT: Under the influence of caffeine, an increase was also noted while standing, and, during exercise, particularly at trot, the increase was exaggerated (e.g., No. 1 and No. 3 at 5.0 g). The recovery in heart beat after the exercise was delayed (Figure 5). In order to examine the pattern of increase in heart beat during running under the influence of caffeine, a control experiment was carried out, in which No. 3, which showed the most notable reduction of lap time, to 13.8 sec, was so driven after an injection of 2.5 g caffeine that an average lap time would become 13.0 sec. When the changes in heart beat in the two tests were compared, a factor that could be attributed to the drug was found (Figure 6).

No specific change attributable to the drug was observed with regard to the range of variation in the R-R intervals, the course of fluctuation, number of gaits, respiration rate, etc.

DISCUSSION

The changes in heart beat, respiration rate and behavior in the horses at rest caused by the caffeine were mild. However, the effect of the drug was manifest in the horses in motion, with increased forwarding capacity and heart beat. The results of this experiment clearly demonstrate the tendency of caffeine to accelerate the forwarding ability of a horse. The effect of the drug in the previous experiment, on trans- oxocamphor, and that of caffeine on lap time were compared using the experimental horse (No. 3) which was used in the present

experiment and the previous experiment (Figure 7). The usual dose of trans-α-apocamphor (200 mg/head, i.v.) produced no appreciable change in lap time, whereas sodium benzoate solution of caffeine produced a notable effect at 1/2 its usual dose (5.0 g/head, s.c.), indicating its strong doping effect. MacKay (4) reported that 10 grains (about 0.7 g) of caffeine enhanced the impulse of a horse and its reactivity toward the whip, but because of insufficient description of the experimental procedure, the detail of the experiment is not clear. He generalized the effects of caffeine as diversified, including the reduction of the reaction time, improvement of coordination, control of fatigue, etc. (7). Perhaps, the accumulation of these effects brings forth the enhancement of forwarding capacity.

With regard to the effect of caffeine on the circulatory function during exercise, Margaria et al. (5) failed to observe any change in the maximum heart beat on the all-out run on a treadmill. According to Ganslen et al. (2), who conducted a treadmill test (normal walk at 91.5 m/min) using human subjects, no appreciable change was produced by an injection of caffeine or pentetrazol alone, but a combination of the two drugs resulted in lower heart beat, higher systolic pressure, and more favorable endurance. Because of the drop in heart beat and elevation of systolic pressure, the drug was regarded as having improved the blood circulation during the exercise. In horses, the heart beat was increased by caffeine, but a reduction of lap time corresponding to the increment of heart beat did not necessarily occurred at the same time. Therefore, although the stimulatory effect of caffeine on heart beat, increasing the value above the level in accordance with the intensity of exercise, was confirmed, the significance of the increment of heart beat in relation to the exercise is yet undetermined. Under the influence of caffeine, a series of atrioventricular or sinoauricular irregularities suggesting a disturbance in excitation transmission were indicated, showing the lack of smooth control of the circulatory system during a shift from

motion to a stationary state. Such disturbance is likely to cause unexpected accident. Accidental deaths of human individuals due to a dope have frequently reported (6), and the prohibition of dope is strongly advocated from a medical standpoint. The doping of race horses has been criticized more or less from a moral standpoint. The result of thepresent experiment indicate the prohibition of dope even for the physical well-being of the animals.

SUMMARY

In order to observe the effect of caffeine on the running performance of horse, sodium benzoate-caffeine was hypodermically injected at 2.5 and 5.0 g per head to 3 thoroughbred riding horses, and changes in lap time, ECG wave pattern, heart beat, R-R interval, number of gaits, and respiration rate (during stna before exercise and at recovery) were studied. As a preliminary experiment, the effect of caffeine on horses at rest was also studied.

1. Caffein increased the forwarding ability of the horses.

2. Caffeine intermittently produced a series of arrythmic signs, including second atrioventricular blocks and sinoauricular blocks immediately after the suspension of motion.

3. Caffeine increased the heart beat during motion, particularly at trot, and delayed the recovery of heart beat.

4. Caffeine exerted no specific effect on the course of fluctuation of the R-R interval system, number of gaits, or respiration number.

5. Caffeine produced significant changes during the exercise even at a dose at which no change was produced in the preliminary experiment.

6. Caffeine produced more notable effect on the forwarding ability of the horses than trans-π-oxocamphor.

The use of dope should be prohibited for the physical well-being of the horses, with due consideration of the arrythmic signs shown by the horses under the influence of caffeine, immediately after the suspension of exercise.

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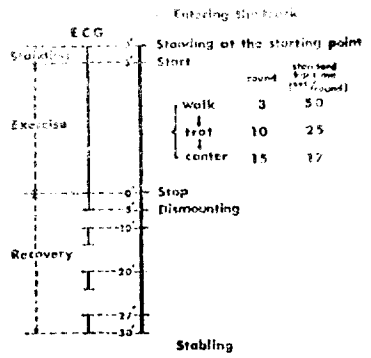
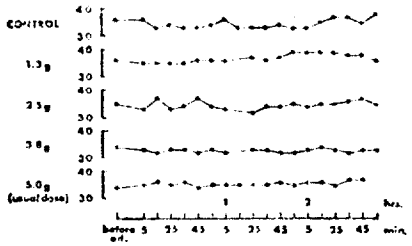
Fig. 1. Experimental Procedure



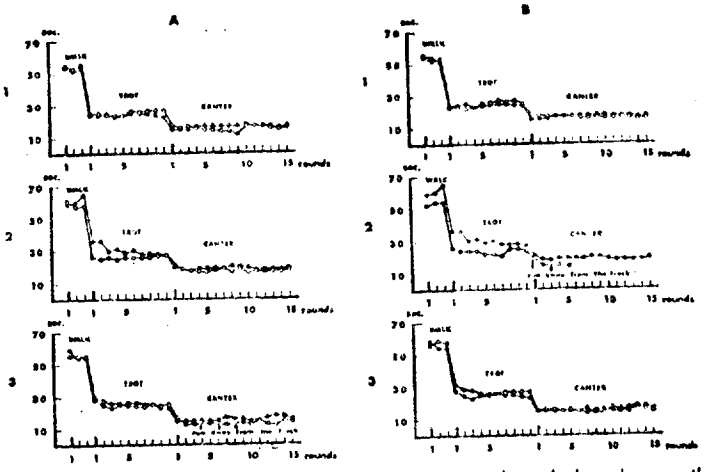
Remarks: 1. The circular track used was 30 m in diameter. 2. The standard lap times were chosen empirically. 3. Drugs were administered about 1 hour before the start.

Fig. 2. Heart Rate at Rest



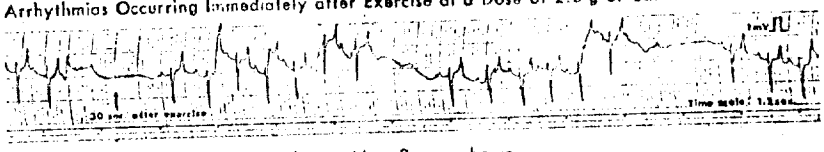
Remarks: Data obtained from horse No. 1 are shown. The ordinate indicates the heart rate per minute and the abscissa the time after administration. before ad.: before administration.

Fig. 3. Lap Time



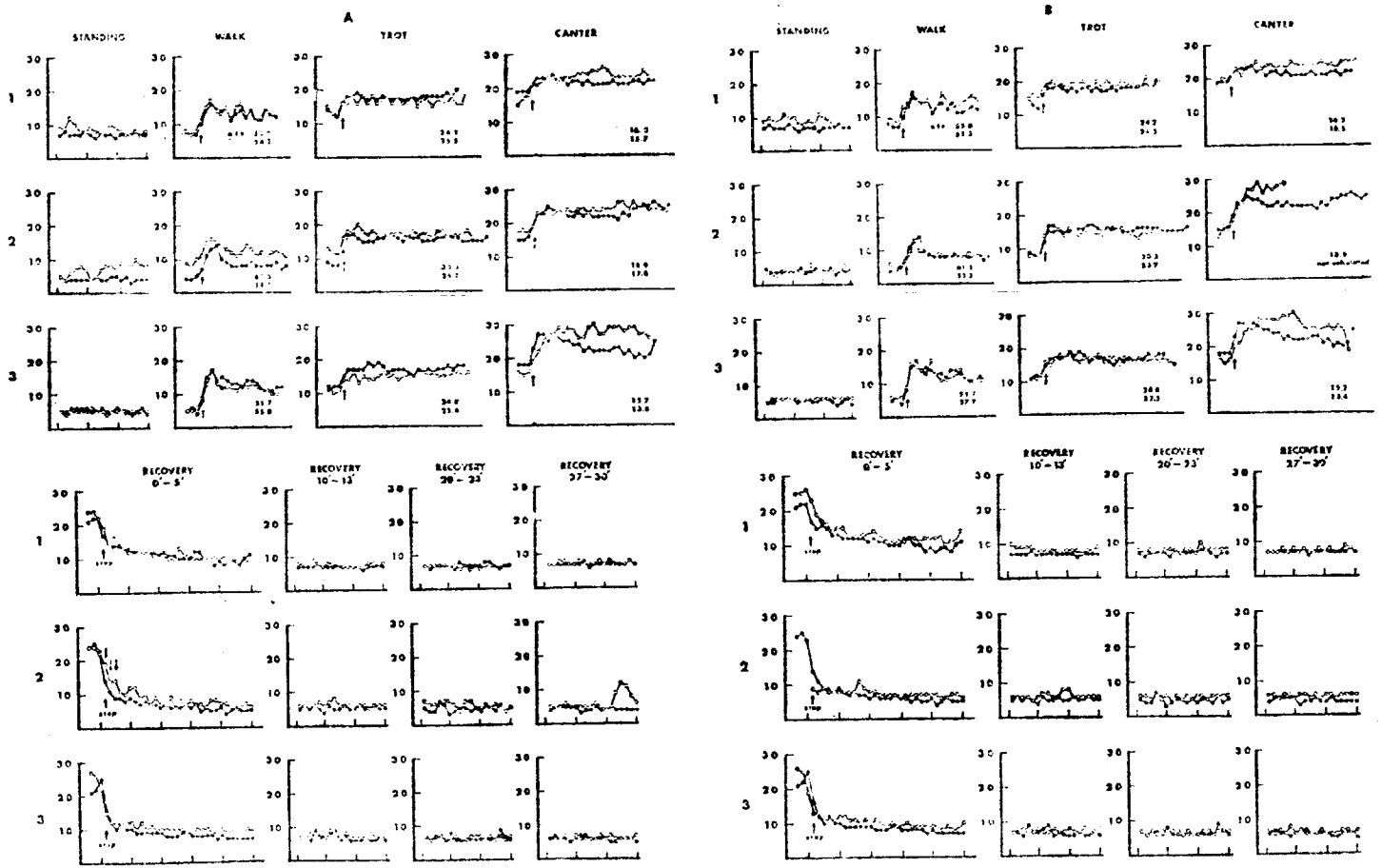
Remarks: The ordinate indicates the lap time in seconds and the abscissa the number of rounds. 1, 2, and 3: The numbers of the experimental horses. ●: control. ○: caffeine and sodium benzoate. A: 2.5 g. B: 5.0 g. ☆: An extreme enhancement of the running performance occurred after administration of caffeine in this case. It became impossible to continue running at canter on an experimental track of 30 m in diameter, because the diameter was too short for a horse to run at an enhanced speed. ↓: Run away from the track.

Fig. 4. Arrhythmias Occurring Immediately after Exercise at a Dose of 2.5 g of Caffeine and Sodium Benzoate



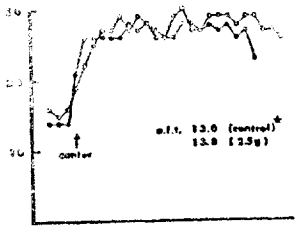
Remarks: ECGs obtaind from horse No. 2 are shown.

Fig. 5. Heart Rate per 10 Seconds during Exercise and Recovery



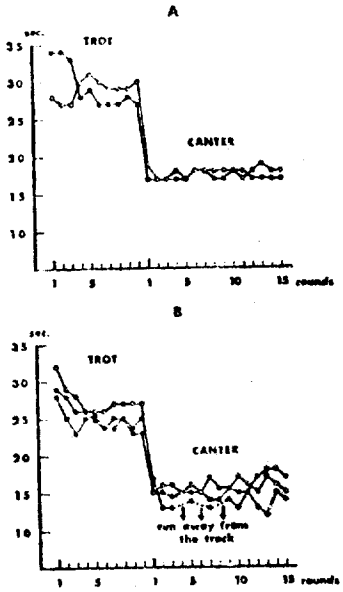
Remarks: The ordinate indicates the heart rate per 10 seconds and the abscissa the number of periods of 10 seconds each in order. 1, 2, and 3: The numbers of the experimental horses. C: control. C: caffeine and sodium benzoate. A: 2.5 g. B: 5.0 g. [illegible] average lap time in seconds (the upper: control; the lower: caffeine and sodium benzoate). ★: See Fig. 2. ↓: arrhythmias. See Fig. 4.

Fig. 6. Increase in Heart Rate at Canter Due to Caffeine and Sodium Benzoate



Remarks: Data obtained from horse No. 3 are shown. ★: A control experiment with 13.0 seconds as average lap time at canter was carried out as supplement. The ordinate indicates the heart rate per 10 seconds and the abscissa the number of periods of 10 seconds each in order. ●: control. ○: caffeine and sodium benzoate, 2.5 g. a.l.t.: average lap time in seconds.

Fig. 7. Comparison of Influence upon the Running Performance between *trans*-π-Oxocamphor and Caffeine and Sodium Benzoate



Remarks: Data obtained from horse No. 3 are shown. The ordinate indicates the lap time in seconds and the abscissa the number of rounds. ●: control. A: *trans*-π-oxocamphor (○: 200 mg, a usual dose). B: caffeine and sodium benzoate (◇: 2.5 g; ○: 5.0 g, a usual dose).

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競走馬における doping drugs の薬理学的研究

II. Caffeine

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(昭和46年7月22日受付)

緒　　言

コーヒー，コーラなどのカフェインを含む飲料やカフェイン製剤を与えて，競技成績を向上させようという試みが古くから行なわれ[6]，それに関連した研究も多い[7]．競走馬の doping でも，カフェインは最も高い陽性率を示すものの一つである．しかしカフェインと馬の運動能力との関連を，実際に実験的に観察したものは極めて少なく，わずかに MACKAY[4] の簡単な報告がみられるにとどまっている．著者らは前報の *trans*-π-oxocamphor[1] に引き続いて，カフェインが馬の運動に及ぼす影響を観察したので，その概要を報告する．

実験材料および方法

実験馬：アパロン乗馬学校所属のサラブレッド種乗馬3頭（せん，6才，体重約420～500 kg）を使用した．1968年1月24日～5月7日（温度7.0～18.0°C，湿度39～88%）の期間，予備実験として安静馬における投与実験を行ない，引き続いて同年5月15日～6月19日（温度17.5～26.0°C，湿度36～73%）の期間，運動負荷実験を行なった．

薬物：もっとも一般的なカフェイン製剤として，局方20%安息香酸ナトリウムカフェイン（以下アンナカと略す）注射液を選び使用した．投与量は，安静馬における実験では，アンナカ1頭当たり1.3 g，2.5 g，3.8 g および5.0 g（常用量[3]）の4種類，運動負荷実験では2.5 g および5.0 g の2種類とし，すべて皮下注射とした．いずれの実験においても，対照はアンナカ5.0 g 投与と同容積の25 ml 生理食塩液とした．投与は，安静馬における実験では1週間に1回とし，低い量から始めて順次増量し，運動負荷実験では1週間1回投与とし，できるだけ無作為的な配列となるように心がけた．

運動負荷：前報[1] と同様の方法によった（Fig. 1）．ただし標準 lap time（前報における標準速度）を並歩50 sec./周（114 m/min.），速歩25 sec./周（228 m/min.），駈歩17 sec./周（335 m/min.）に変更した．なお3号馬についてのみ，駈歩13sec./周（438 m/min.）を標準とする対照実験を追加した．また運動負荷は薬物投与約1時間後に行ない，それまでは馬房内に繋留した．

観察項目：安静馬における実験では，馬房内で左右から長くかつ緩く手綱を張って，馬の頭部のみを緩く保定して行なった．投与30分前から投与3時間後まで，A-B 誘導による心電図を，無線遠方測定法を用いて連続記録し，呼吸数を視診により約10分間隔で計測し，体温（2号馬についてのみ実施）を投与前1回，投与後は約30分間隔で測定した．また behavior の変化を随時記録した．

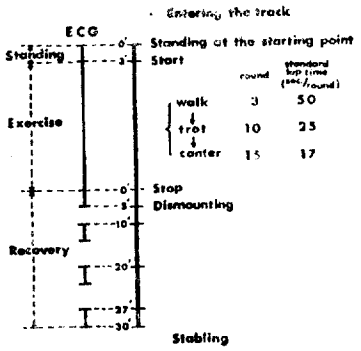
運動負荷実験では心電図記録，lap time 計測および騎乗者の内省報告の聴取など，前報[1] と同様に行なった．さらに今回から1周当たりの歩数（完歩数）の計測と，運動前の立馬時，運動停止の直後，回復3・5・10・15・20分および30分に呼吸数を計測した．

実験結果

1．安静馬における投与実験

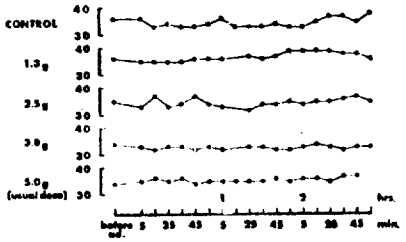
投与量3.8 g 以上において，心拍数と behavior に軽微な変化が観察された以外には，薬物による変化を認めなかった．対照では，心拍数は時間の経過とともに漸減するか，あるいは減少してふたたび投与前の値にまで増加し，また減少するとい

Fig. 1. Experimental Procedure



Remarks: 1. The circular track used was 30 m in diameter. 2. The standard lap times were chosen empirically. 3. Drugs were administered about 1 hour before the start.

Fig. 2. Heart Rate at Rest

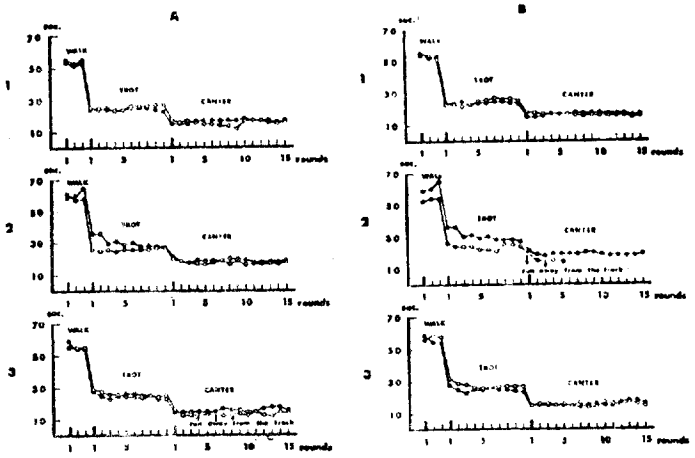


Remarks: Data obtained from horse No. 1 are shown. The ordinate indicates the heart rate per minute and the abscissa the time after administration. before ad.: before administration.

った周期的変動を示した．投与量3.8 g以上では，このような変化が消失し，投与前の値をそのまま維持する場合が多かった (Fig. 2)．また対照では頭頸部を低く垂れ，手綱に頭頸部の重量をもたせかけて佇立し，いわゆる drowsiness と思われる状態が間歇的に出現した．しかし 3.8 g 以上の投与において，早いもので15分，遅くも約1時間でこのような drowsiness の発現がとまった．アンナカが激しい興奮を起こすことはなかったが，ただ 3.8 g 投与の1例 (2号馬) で，投与後6～13分の間激しい運動興奮を示し，その後引き続いて3時間まで音に敏感であり，また時折り精神的興奮によると思われる高いいななきを頻発した．しかし約1ヵ月後に行なった追試時には，drowsiness の消失がみられたのみであり，運動興奮も精神的興奮も再現されなかった．また 5.0 g の投与でも drowsiness が消失するにとどまった．

以上の結果を参照して，4種の投与量のうちか

Fig. 3. Lap Time



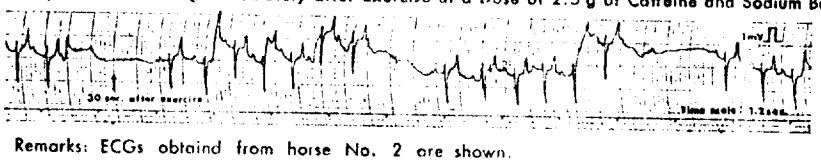
Remarks: The ordinate indicates the lap time in seconds and the abscissa the number of rounds. 1, 2, and 3: The numbers of the experimental horses. ●: control. ○: caffeine and sodium benzoate. A: 2.5 g. B: 5.0 g. ★: An extreme enhancement of the running performance occurred after administration of caffeine in this case. It became impossible to continue running at canter on an experimental track of 30 m in diameter, because the diameter was too short for a horse to run at an enhanced speed. ↓: Run away from the track.

ら，安静時の投与において変化を認め難い量として2.5 g，変化が認められる量として 5.0 g の 2 種を選び，運動負荷実験での投与量とした．また投与 1 時間後に運動負荷を行なうことにきめた．

2. 運動負荷実験

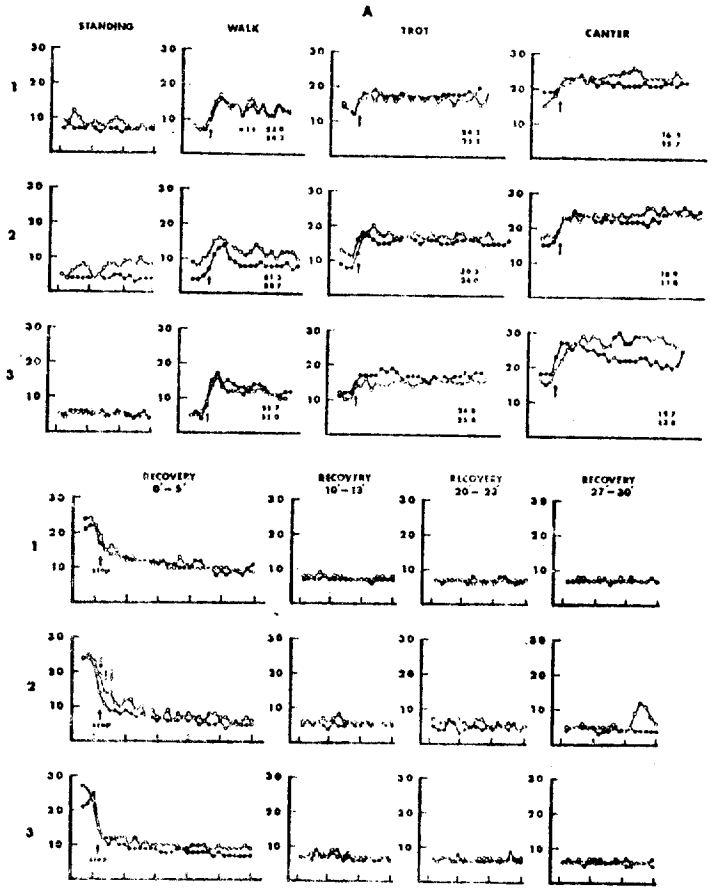
Lap time：アンナカによって馬の前進能力が増進し，あらかじめ指定された標準 lap time を維持しようとする騎乗者の扶助にもかかわらず，lap time が短縮した (Fig. 3)．この lap time の短縮は，安静馬での実験では変化がとらえ難かった 2.5 g でも起こった．たとえば 1 号馬の駈歩第 4～9 周，2 号馬の駈歩第 1～5 周のように，lap time が漸次短縮した．ことに 3 号馬では速度が増加し過ぎたため，一旦走路から出て巻乗りを行ない，態勢を立て直して，ふたたび走路に進入しなければならなかった．投与量5.0 g では，2号馬のように，直径の小さい円型走路での運動を放棄した場合もみられた．また騎乗者によれば，アンナカ投与時どの歩様でも前進意欲の向上が感じられ，運動が強くなるほど，標準 lap time への

Fig. 4. Arrhythmias Occurring Immediately after Exercise at a Dose of 2.5 g of Caffeine and Sodium Benzoate

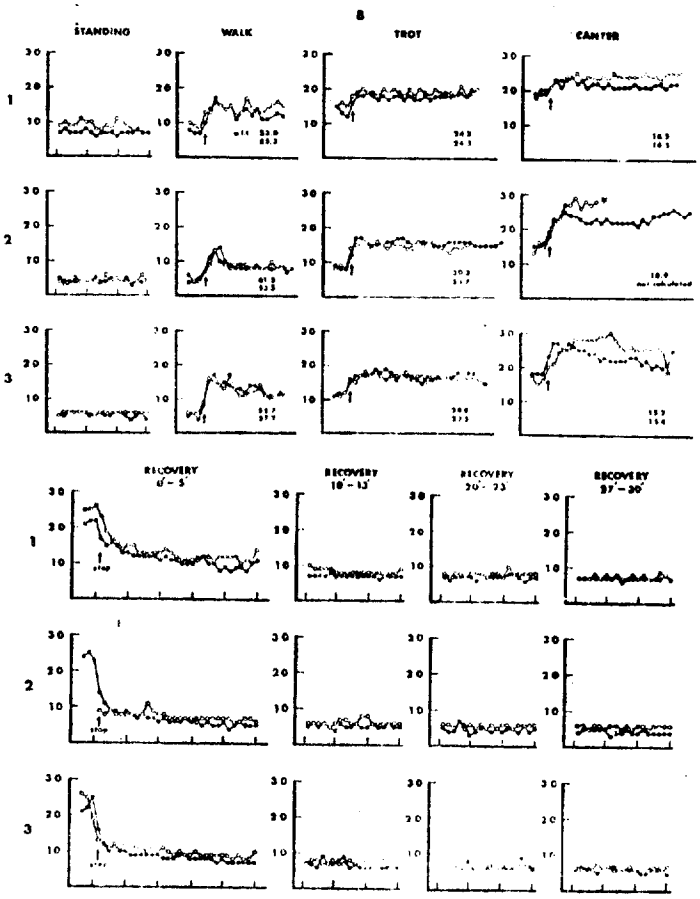


Remarks: ECGs obtaind from horse No. 2 are shown.

Fig. 5. Heart Rate per 10 Seconds during Exercise and Recovery



(Fig. 5 続)



Remarks: The ordinate indicates the heart rate per 10 seconds and the abscissa the number of periods of 10 seconds each in order. 1, 2, and 3: The numbers of the experimental horses. ●: control. ○: caffeine and sodium benzoate. A: 2.5 g. B: 5.0 g. a.l.t.: average lap time in seconds (the upper: control; the lower: caffeine and sodium benzoate). ★: See Fig. 3. ↓: arrhythmias. See Fig. 4.

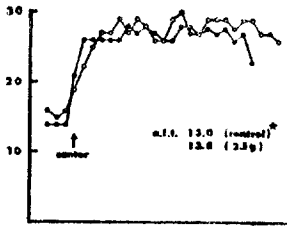
調節が困難となり，かつ馬の運動に弾撥力が増加したと報告された．

心電図波形：2号馬 2.5 g 投与時，運動を終了した直後に第2度房室ブロックが相ついで現われ，あるいはさらに，洞房ブロックが継起するといった一連の不整脈が3回にわたって出現した (Fig. 4)．このほかには，異常な心電図を認めなかった．

心拍数：アンナカ投与下では，運動前の立馬時すでに増加を示したものがあり，運動時，なかでも駈歩時に増加が目立ち（たとえば 5.0 g の1・3号馬），また運動後の心拍数の回復が遅れた (Fig. 5)．薬物による運動時の心拍数の増加については，2.5 g のアンナカ投与によって，平均 lap time が 13.8 sec. というもっとも目立った短縮を示した3号馬について，平均 lap time が 13.0 sec. になるように激しい扶助で馬を追い込む対照実験を改めて行なった．そして心拍数の変化を相互に比較した結果，その増加には，薬物自体に起因するものが含まれていることを確かめた (Fig. 6)．

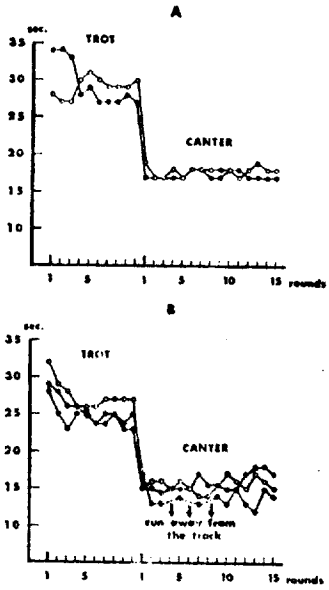
その他，R-R 間隔時系列の変動幅・変動経過，平均，時間的な変化では，薬物によると考えられる特定の変化を認めなかった．

Fig. 6. Increase in Heart Rate at Canter Due to Caffeine and Sodium Benzoate



Remarks: Data obtained from horse No. 3 are shown. ★: A control experiment with 13.0 seconds as average lap time at canter was carried out as supplement. The ordinate indicates the heart rate per 10 seconds and the abscissa the number of periods of 10 seconds each in order. ●: control. ○: caffeine and sodium benzoate, 2.5 g. a.l.t.: average lap time in seconds.

Fig. 7. Comparison of Influence upon the Running Performance between *trans*-π-Oxocamphor and Caffeine and Sodium Benzoate



Remarks: Data obtained from horse No. 3 are shown. The ordinate indicates the lap time in seconds and the abscissa the number of rounds. ●: control. A: *trans* π-oxocamphor (○: 200 mg, a usual dose). B: caffeine and sodium benzoate (◆: 2.5 g; ○: 5.0 g, a usual dose).

考　　察

カフェインによって安静馬に起こる変化は，心拍数，呼吸数，behavior とも概して軽微であった．しかし安静時には変化らしい変化を認め難かった量でも，運動時には薬物の影響が強く現われ，前進能力が目立って増進し，心拍数の増加も明瞭となった．カフェインが馬の前進能力を強く増進することについては，この実験の結果から明らかである．前報の *trans*-π-oxocamphor の実験と，今回の実験とに，たまたま共通して使用された実験馬 (3号馬) について，両薬物が lap time に及ぼす影響を比べてみた (Fig. 7). *trans*-π-oxocamphor の常用量 (200 mg/head, i. v.) では，lap time の変化は目立たない．これに対してアンテカでは，常用量 (5.0 g/head, s. c.) の 1/2 量ですでに明らかな影響が現われ，その強い doping 効果がうかがわれる．MACKAY[4] も10グレイン (約 0.7 g) のカフェインが馬の推進力を増加し，また鞭にもよく反応するようにさせたと報告しているが，実験方法等の記載が十分でなく，その詳細は不明である．カフェインは反応時を短縮し，あるいは共調 (coordination) を改善し，また疲労の発現をおさえるなど，多彩な効果を示すと総説[7] されている．これらの効果の集積が，前進能力の増進をもたらすのであろう．

運動中の循環機能に及ぼすカフェインの影響について，MARGARIA ら[5] は，トレッドミル上のオールアウト走の最高心拍数に変化を与えなかったと述べている．GANSLEN ら[2] も，人のトレッドミル・テスト (91.5 m/min. の常歩) において，カフェインあるいはペンテトラゾールの単独使用では，なんら変化が現われなかったが，両薬物を併用すると，対照よりも低い心拍数で，かつ高い収縮期血圧で，テストに耐過したと報告している．この場合，心拍数の減少と同時に収縮期血圧の上昇が起こっていることから，薬物が運動時の血行動態を改善したものと推測している．馬では，カフェインによって心拍数は増加したが，心拍の増加分に見合った lap time の短縮が，必ずしも同時に起こっているというわけではなかった．従ってカフェインが，運動強度に見合った水準以上の心拍増加をもたらしたとみなされるが，このいわば余分の心拍増加が，運動にどのような意義をもつのか，不明である．他方カフェイン投与下で

は，運動停止の直後に，房室間および洞房間に一連の興奮伝導障害が反復し，動から静への転換時における循環系の調節に円滑を欠く場合がみられた．このような興奮伝導障害が，不測の事故を招く可能性も十分考えられる．人では doping による多くの事故死が知られており[6]，医学的見地からもその禁止が強く支持される．これに対して競走馬における doping は，従来ややもすれば，不正行為に対する道徳的見地から禁止が叫ばれる傾向が強かった．このときに，本実験の結果は，馬の健康保全の面でも，当然 doping を禁止すべきであるという示唆を与えるものである．

要　　約

カフェインが馬の運動に及ぼす影響を観察するため，サラブレッド種雄馬3頭を用いて，アンプカ1頭当たり 2.5 g および 5.0 g を皮下注射し，運動負荷時に起こる lap time・心電図波形・心拍数・R-R 間隔・歩数・呼吸数（運動前の立馬時および回復時のみ）の変化を観察し，次の結果を得た．なお予備実験として，安静馬におけるカフェインの影響をも観察した．

1. カフェインは，馬の前進能力を増進した．

2. カフェインは，運動停止の直後に，第2度房室ブロックおよび洞房ブロックを含む一連の不整脈を，間歇的に出現させることがあった．

3. カフェインは，運動時，特に駈歩時に心拍数の増加をもたらし，また心拍数の回復を遅らせた．

4. カフェインは，R-R 間隔時系列の変動経過・歩数・呼吸数には，特定の影響を及ぼさなかった．

5. 安静時には変化を起こし難いカフェイン量でも，運動負荷時には大きな変化をもたらした．

6. カフェインは，*trans-π*-oxocamphor に比べて，前進能力に与える影響は顕著であった．

以上の結果について，doping の立場から考察し，特にカフェイン投与下で起こった運動停止直後の不整脈を重視し，馬の健康保全のためにも，doping 行為を禁止すべきであると考察した．

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EFFECT ON THE OFFSPRING OF REPEATED CAFFEINE ADMINISTRATION TO PREGNANT RATS

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Summary. Repeated intraperitoneal injections of caffeine given to rats in dosages ranging from 4 to 16 mg daily throughout pregnancy resulted in significant resorptions and decrease in the birth weight of the offspring. These effects were dose-related. Developmental malformations, however, were not observed in the offspring at these dosage levels.

These results indicate that caffeine, even in low doses which at the lower levels of the experiment may approximate to the daily human intake of a heavy coffee consumer, suppresses growth of embryonic and fetal tissues.

INTRODUCTION

Since caffeine is a universal beverage and is consumed in large quantities by the human race, its effect during gestation on the developing offspring is important. There is good evidence that caffeine is mutagenic in bacteria (Demerec and his co-authors, 1950; Demerec, Bertani & Flint, 1951; Gezelius & Fries, 1952; Novick, 1955; Greer, 1968), fungi (Fries, 1950; Zetterberg, 1959) and plants (Kihlman & Levan, 1949; Kihlman, 1952), as well as in *Drosophila* (Andrew, 1959; Ostertag, 1963; Ostertag & Haake, 1966; Mittler, Mittler & Owens, 1967) and in human cells *in vitro* (Ostertag, Duisberg & Stürmann, 1965; Ostertag, 1966a, b; Ostertag & Greif, 1967). Although there have been some conflicting reports on the mutagenicity of caffeine in higher organisms (Adler, 1970; Epstein, 1970), chromosomal breakage has been induced in human cell cultures as well as in *Drosophila* (Kuhlmann, Fromme, Heege & Ostertag, 1968).

Caffeine has been proven to be teratogenic in the offspring of mice following a single large intraperitoneal injection (Nishimura & Nakai, 1960; Fujii & Nishimura, 1969), but the effect of relatively low doses has not been studied. In the present paper, the effect of repeated caffeine injections on the offspring of pregnant rats given a dose approximating to that of a high daily coffee consumption of humans is examined.

MATERIALS AND METHODS

Forty female Holzer rats, each weighing approximately 200 g, were mated. Following observation of spermatozoa in the vaginal smears, each rat was given intraperitoneal injections of caffeine at 6-hr intervals. Taking the figure of 100 mg caffeine in an average cup of coffee, the daily equivalents per 50 kg (the average weight of an adult human female) were calculated from the dosage schedule shown in Table 1.

Table 1. Dosage schedule of caffeine given to mated female rats

*Group**	*Caffeine†*	*Total daily dose*	*Daily caffeine equivalent as coffee/50 kg*
A	1 mg	4 mg	10 cups
B	2 mg	8 mg	20 cups
C	4 mg	16 mg	40 cups
D (control)	—	—	—

* Each group contained ten rats.
† Given intraperitoneally every 6 hr; the control group received 1 ml normal saline intraperitoneally every 6 hr.

The dosage was continued from Day 0 to Day 20, at which time all animals were killed. Random serum caffeine levels were determined by the method of Axelrod & Reichenthal (1953) in each group. The peak of caffeine concentration occurred 30 min after caffeine injection. The highest serum levels of caffeine reached in the experimental groups were: Group A, 2·8 mg/100 ml; Group B, 6·6 mg/100 ml; Group C, 14·6 mg/100 ml.

The litter size, number of resorptions, fetal weights and placental weights were determined. The fetuses were fixed in 10% buffered formalin and examined for anomalies. Attempts to identify skeletal malformations were made by staining in alizarine-KOH and clearing soft tissues in glycerol.

RESULTS

Table 2 summarizes the average litter size, number of resorptions, fetal weight and placental weight in each group. A 20% decrease in litter size was observed in Group A given 4 mg caffeine/day and 40% decrease in Groups B and C.

Table 2. Effects of caffeine administration on the offspring of pregnant rats

Group	*Daily dose of caffeine (mg)*	*Average litter size*	*Average no. of resorptions*	*Average fetal weight (g)*	*Average placental weight (g)*
A	4	8	2·1	4·20	0·6
B	8	6	4·0	3·50	0·5
C	16	6	4·0	3·20	0·4
D	Control	10	1·2	4·60	0·7

Likewise, the average number of resorptions was twice as great in Group A (4 mg caffeine/day) as compared with the control group, and was quadrupled in Groups B and C.

The average fetal weight was significantly decreased in all experimental groups, the decrease appearing to be dose-related. The placental weight generally corresponded to the weight of the fetus and the placentae showed no significant gross or histological changes.

No significant developmental malformations or skeletal defects were observed in the fetuses after dissection and staining.

DISCUSSION

Caffeine is a purine–1,3,7-trimethyl xanthine. It has been observed recently that inhibition of DNA polymerase activity is effected in human cell cultures at concentrations of caffeine above 500 μg/ml after 48 hr (Wragg, Carr & Ross, 1967). When 1% caffeine is added to Hela cell cultures, extensive changes in the fine structure of the cells occurs with expulsion of nuclear material into the cytoplasm (Ostertag & Fromme, 1967). In addition, breakage has been reported in human chromosomes, even in doses below 500 μg/ml (Kuhlmann *et al.*, 1968).

More recently, a high frequency of chromatid aberrations, predominantly of the unstable type, has been induced with caffeine in human leucocytes and human embryonic cells in culture (Lee, 1971). Studies on the fate of radiation-induced chromosome aberrations (Buckton, Jacobs, Court Brown & Doll, 1962; Norman, Sasaki, Ottoman & Fingerhut, 1966; Sasaki & Norman, 1967; Leonard, 1970) have shown that such unstable aberrations do not survive cell divisions due to the genetic imbalance caused by the abnormal distribution of genetic material to the daughter cells. Furthermore, the absence of exchange aberrations and the restriction of damage to the DNA synthesis phase which Lee (1971) observed may relate to the ambiguous results on the effectiveness of caffeine in the induction of dominant lethal mutations in higher organisms.

A change in sex ratio has been observed in female larvae of *Drosophila* exposed to 1% caffeine for 1 hr (Zetterberg, 1959). After hatching, there was a shift of sex ratio in favour of females. Previous investigators have shown for X-rays (Oster, 1958; Ostertag & Muller, 1959; Ostertag, 1963) as well as for chemical mutagens (Ostertag, 1963) that this is related to genetic damage induced by chromosomal breakage. Loss of the X chromosome in male cells is lethal whereas a single X chromosome loss in female cells can be tolerated.

In the dog, Axelrod & Reichenthal (1953) have shown that caffeine is distributed freely in the intracellular water of all tissues. Furthermore, Goldstein & Warren (1962) have demonstrated that caffeine administered to the human adult gains access to the gonads and fetal tissues and reaches concentrations substantially the same as those in the plasma. It can, therefore, be concluded that the consumption of coffee several times daily by the human pregnant female allows constant exposure of the fetal tissues to the effects of the caffeine. Fetal gonadal tissue is also exposed, with the potential hazard of a deleterious effect on the germ cells.

Congenital malformations in the offspring of mice treated with a single large

(250 mg/kg body weight) intraperitoneal injection of caffeine have been observed by Nishimura & Nakai (1960) and Fujii & Nishimura (1969). When the compound was administered between the 9th and 14th days, malformations, predominantly in the skeletal system, were found. Haematomata were also frequently observed.

The present experiments utilizing relatively small dosages of caffeine indicate that although fetal malformations are not produced at this low concentration, there is a significant degree of fetal loss and decrease in birth weight.

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Wakefulness Caused by Caffeine*

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With 1 Figure in the Text

(Received November 26, 1963)

Caffeine is probably the most widely used of all drugs, at least in those parts of the world where coffee is a popular beverage, or where it is the custom to drink large amounts of strong tea. It might be considered self-evident that people drink coffee in order to experience certain psychotropic effects. However, no convincing evidence that this is true has yet been adduced; neither can it be said with assurance just what effects people seek and obtain when they drink coffee, nor whether these are the same for everyone. Moreover, although coffee is equally available to all, and although coffee-drinking is socially acceptable (except in limited circles), people differ greatly in their habitual consumption of this beverage. Qualitative or quantitative differences in sensitivity to psychotropic actions of caffeine might possibly underlie the wide individual differences in coffee-drinking habits.

An extensive body of experimentation on the psychotropic effects of caffeine has been reviewed recently (WEISS and LATIES 1962). The drug's actions are peculiarly subtle. The most firmly established effect of ordinary doses (150—300 mg, the amount in 1—2 cups of strong coffee) is to counteract the decrement in performance of numerous psychomotor tasks which is caused by fatigue. Improvement in performance above control levels is rarely achieved. Thus a major action of caffeine appears to be a modification of the state of wakefulness, to which action many of its other effects may be ascribed.

This rather special kind of stimulant effect is reflected in the general use of coffee as an aid to wakefulness, and in the corollary belief that caffeine disturbs sleep. This belief, however, is far from unanimous. Some people assert that even a single cup of coffee in the evening makes them wakeful, others hold that they are undisturbed by coffee or even that it

* Dedicated to my teacher, colleague, and friend, Professor OTTO KRAYER, on the occasion of his sixty-fifth birthday. A preliminary report of this work has been published (GOLDSTEIN and WARREN 1961).

helps them to sleep. Such contradictory views may only mean that people's beliefs are not well grounded in fact, but they could also reflect real quantitative or qualitative differences in individual responses to caffeine.

In this paper we examine effects upon sleep and upon withdrawal headache. We could answer our questions sufficiently well by means of a relatively crude instrument, a self-evaluation questionnaire filled out by subjects after the night's sleep in their usual surroundings. For this kind of experimental procedure medical students proved to be very satisfactory subjects.

Methods

The four experiments reported here were conducted as teaching exercises designed to illustrate the principles of the clinical trial (HILL 1960). Each experiment was with a different class of medical students, one (exp. a) at Harvard (November 1954), three at Stanford (October 1959, January 1960, April 1960). There were 230 subjects, nearly all males 22—26 years of age. Practically all students in each class participated at the outset, but in experiments of two or four nights duration some failed to complete the series.

The basic procedure was administration of caffeine or a lactose placebo before bedtime. Criteria of effect were responses to a questionnaire filled out the next morning. Subjects were asked to estimate how long it took them to fall asleep and how soundly they slept, and to state whether or not they experienced a headache on awakening. Exact wording of the questions is reproduced in the relevant tables of data. Each subject was also queried about his habitual daily caffeine intake, and about whether or not he believed that coffee disturbs his sleep.

In exp. a, identical capsules contained lactose, pentobarbital (150 mg), or caffeine (200 mg). In the other experiments samples of soluble decaffeinated coffee (Sanka brand, 2.5 g) were used, to which were added caffeine (150 mg) or an amount of lactose sufficient to produce a perfect color match. In order to match color and consistency it was found necessary to screen the caffeine powder (250-mesh) before blending, and glass vials were used (not plastic) to prevent caffeine from sticking to the walls. Preliminary trials with 50 experienced coffee-drinkers demonstrated that our samples containing caffeine could not be distinguished by taste or appearance from the placebos. Subjects were instructed to prepare the "coffee sample" to their taste with hot water (cream and sugar if desired) and drink it all.

All the usual precautions of double-blind design were followed rigorously. Samples were identified only by serial number or by student names, and assignments were made with the aid of a table of random numbers. In exps. b, c, and d, a balanced crossover was employed, lactose-caffeine and caffeine-lactose in the 2-night study, lactose-caffeine-caffeine-lactose and caffeine-lactose-lactose-caffeine in the 4-night studies; assignment of subjects to one or the other order of administration was randomized.

Certain further points of difference between the experiments deserve brief mention. Exp. a purported to deal with hypnotic agents. Similar teaching exercises with previous classes (GOLDSTEIN 1953) led the subjects to expect a barbiturate or placebo, and the exercise was entitled "Class Experiment on Hypnotics". A capsule was ingested one-half hour prior to subject's usual bedtime. There were no restrictions on the intake of caffeinated or other beverages on the day of the experiment. Information about habitual caffeine intake was obtained after all the data

had been collected. Exp. b also purported to deal with hypnotic agents. The instructions stated: "This is a clinical trial on the effects of a drug upon sleep. The drug has been added to one of your coffee samples and an inert material to the other." A sample was ingested one hour before the usual bedtime on each of two successive nights. All beverages were prohibited after supper on experimental days. Exps. c and d were presented frankly as attempts to gain information about the effects of caffeine on sleep. The "coffee samples" were taken one hour before bedtime on each of four successive nights, and all beverages were prohibited after lunch on experimental days. In exp. d, on the first night, subjects were given a known caffeinated sample as a standard of comparison for the unknowns administered on the subsequent four nights.

Statistical analyses of measurement data were performed by Student's *t*-test, of ordinary contingency tables by Chisquare, of ordered contingency tables by the Wilcoxon 2-sample rank test (GOLDSTEIN 1964).

Results

Responses to "Estimated time to fall asleep (minutes after drug)" are shown for exp. a as a histogram in Fig. 1. Pentobarbital at this dose did not significantly shorten the time required to fall asleep, presumably because these students (in contrast to patients with insomnia) fell asleep so promptly anyway (cf. lactose subjects). In contrast, subjects receiving caffeine were kept awake. The mean estimate of wakefulness produced by caffeine (mean caffeine time less mean lactose time) was 33 minutes, the corresponding median estimate was 20 minutes. Skewing to longer times was very prominent, and some subjects were unable to fall asleep for several hours.

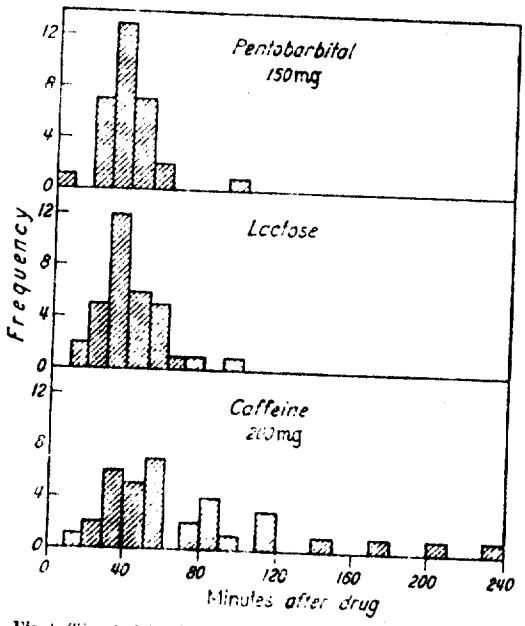

Fig. 1. *Time to fall asleep.* Frequency distribution of subjects in exp. a. For description see text. Times are minutes after drug, which was taken one-half hour before the usual bedtime. *Means and 99% confidence intervals:* Pentobarbital 39 (31—47), lactose 44 (36—52), caffeine 77 (53—101). *Medians:* Pentobarbital 40, lactose 40, caffeine 60. Caffeine differs from lactose or pentobarbital, $P < 0.01$

In exps. b, c, and d, the estimate of time to fall asleep was made simpler. Responses to "How long did it take you to fall asleep (longer time than usual) (about same time as usual) (shorter time than usual)?" are presented in Table 1. Order of administration in the crossover studies had no discernible effect, so the data are all combined in this and subsequent tables. At the 150 mg dose employed here caffeine significantly

Table 1. *Time to fall asleep*

Subjects received lactose or 150 mg caffeine in decaffeinated coffee 1 hour before their usual bedtimes on 2 or 4 nights in a balanced crossover design. Frequencies in the table are responses to "How long did it take you to fall asleep (longer than usual) (about same time as usual) (shorter time than usual)?". In exp. d all subjects ingested a sample containing *known* caffeine on the first night, then the usual blind administration of caffeine and lactose samples on the following 4 nights

	Shorter time	Same time as usual	Longer time	Total
		Exp. b (53 subjects, 2 nights)		
Lactose	8	37	8	53
Caffeine	0	38	15	53
		Exp. c (37 subjects, 4 nights)		
Lactose	10	57	7	74
Caffeine	2	41	31	74
		Exp. d (41 subjects, 4 nights)		
Known caffeine	*1*	*36*	*4*	*41*
Lactose	10	64	8	82
Caffeine	4	53	25	82

Analysis by Wilcoxon 2-sample rank test. Caffeine delayed the onset of sleep, compared with lactose, in all experiments (exp. b, normal deviate $x^* = 2.14$, $P < 0.05$; exp. c, $x^* = 2.59$, $P < 0.01$; exp. d, blind administration, $x^* = 2.91$, $P < 0.01$). Known caffeine (exp. d) did not differ from lactose ($x^* = 0.80$, $P > 0.05$), but fell just short of significantly less effect then blind caffeine ($x^* = 1.63$, $P = 0.052$, 1-tail).

prolonged the time required to fall asleep, as compared with lactose placebo, in all three experiments. The data obtained in exps. c and d were also examined by an intra-subject comparison of caffeine and lactose responses in 22 and 37 subjects, respectively, who completed all 4 experimental nights. Each response was coded as +1 (longer time), 0 (same time as usual), or −1 (shorter time). A score was then computed for each subject, by substracting lactose responses from caffeine responses, and the number of positive and negative scores was analyzed by the sign test. As in Table 1, the hypothesis that caffeine and lactose did not differ in effect on wakefulness was strongly rejected ($P < 0.01$) for each of these experiments. In exp. d, when subjects received known caffeine, they reported only slightly more wakefulness than with lactose, and much less wakefulness than with identical caffeine samples administered on subsequent nights in the blind design.

Effects upon soundness of sleep are shown in Table 2. In exp. a, pentobarbital improved the soundness of sleep and caffeine disturbed sleep, as compared with lactose. The striking effects of caffeine here and in Fig. 1 are especially noteworthy in the context of a hypnotic experiment, in which subjects would be reluctant to attribute wakefulness to a

Table 2. *Soundness of sleep*

Subjects in exp. a received lactose, 150 mg pentobarbital, or 200 mg caffeine by capsule one-half hour before their usual bedtimes on a single night. Subjects in exps. b and c received lactose or 150 mg caffeine in decaffeinated coffee 1 hour before their usual bedtimes on 2 or 4 nights in a balanced crossover design. Frequencies in the table are responses to "Did you sleep more soundly than usual (about as soundly as usual) (less soundly than usual)?"

	Slept more soundly	Slept about as soundly as usual	Slept less soundly than usual	Total
Exp. a (99 subjects, 1 night)				
Pentobarbital	13	18	0	31
Lactose	3	27	3	33
Caffeine	2	18	15	35
Exp. b (53 subjects, 2 nights)				
Lactose	9	34	10	53
Caffeine	4	32	17	53
Exp. c (37 subjects, 4 nights)				
Lactose	6	60	8	74
Caffeine	4	53	17	74

Analysis by Wilcoxon 2-sample rank test. Exp. a Pentobarbital subjects slept more soundly than lactose subjects ($x^* = 2.40$, $P < 0.05$); caffeine subjects slept less soundly than lactose subjects ($x^* = 2.60$, $P < 0.01$). Exp. b Caffeine interfered with soundness of sleep, compared with lactose ($x^* = 1.65$, $P < 0.05$, 1-tail). Exp. c Sleep was less sound on caffeine nights than on lactose nights, but difference not significant ($x^* = 1.43$, $P < 0.05$)

presumed hypnotic drug or placebo. Several subjects wrote unsolicited comments on the extreme degree of wakefulness they experienced, even suggesting that a stimulant drug might have been given in error! At the 150 mg dose in exps. b and c caffeine also acted in the direction of disturbing the soundness of sleep, but the effect appears to be less marked than upon falling asleep, and in exp. c the comparison with lactose just failed of significance.

Coffee accounted for practically all the habitual caffeine intake of our subjects; tea, Coca-Cola, and similar caffeine-containing beverages were taken only occasionally (moreover these contain only about one-quarter as much caffeine

Table 3. *Habitual caffeine intake*

Data are frequencies for all subjects who replied to questionnaire in the 3 experiments in which such information was sought. An average cup of coffee contains about 130 mg caffeine

	Cups of coffee per day				
	0	1	2—4	5 or more	Total
Exp. b	6	12	28	12	58
Exp. c	15	11	11	12	49
Exp. d	18	5	22	15	60
Total	39	28	61	39	167
Per cent of all subjects	23%	17%	37%	23%	

Table 4. *Relationship of wakefulness to habitual caffeine intake*
Data are combined responses in exps. c and d to "How long did it take you to fall asleep?" The responses "about same time as usual" and "shorter time than usual" were pooled into a single category as shown. In these experiments all beverages were prohibited after lunch on experimental days

Daily coffee (cups)	Response	Lactose nights	Caffeine nights
0	Longer time than usual	4	18
	Not longer than usual	47	30
1	Longer time than usual	0	13
	Not longer than usual	23	13
2—4	Longer time than usual	7	16
	Not longer than usual	43	31
5 or more	Longer time than usual	4	8
	Not longer than usual	27	28
Total	Longer time than usual	15	55
	Not longer than usual	140	102

Analysis by Chi-square test. Lactose responses can not be said to differ between coffee groups. Caffeine responses differed from lactose responses in all coffee groups except the most habituated ($\chi^2 = 10.9, 13.2, 4.3, 0.4$). Caffeine effect in the most habituated group was less than in the combined 0—1 cup groups ($\chi^2 = 3.3$, $P < 0.05$, 1-tail).

per serving as coffee). Estimates of the habitual caffeine intake, in cups of coffee per day, are given in Table 3. About one-quarter of the subjects never drink coffee, and another 17% drink only one cup daily, usually at breakfast. Another one-quarter of the subjects drink 5 or more cups daily. This distribution conforms well to that reported by ROPER (1948) for a stratified cross-section of Americans. We regard the first two groups as having negligible chronic exposure to caffeine. Those who drink 2—4 cups daily are, in effect, drinking coffee at each meal, as is the social custom. We regard those who drink 5 or more cups daily as habitues of the drug, since they must seek out special between-meals opportunities for drinking coffee.

Table 5. *Relationship of habitual caffeine intake to belief about disturbance of sleep by caffeine*
Date are combined responses (exps. c and d) to "Do you think coffee drunk just before bedtime disturbs your sleep?"

Daily coffee (cups)	Yes	No
0	12	14
1	8	7
2—4	12	21
5 or more	5	22
Total	37	64

Analysis by Chi-square test. A smaller fraction of subjects in the most habituated group than in the combined 0—1 cup groups believed that coffee disturbs sleep ($\chi^2 = 5.2$, $P < 0.05$).

Table 4 reveals that the subjects who habitually drink most coffee are least affected by caffeine (wakefulness criterion), whereas the fre-

quency of response to lactose is essentially the same regardless of daily coffee consumption. Moreover, the subjects in the most habituated group are generally aware of their insensitivity to the sleep-disturbing effect of coffee, as shown by the data of Table 5.

Table 6. *Relationship of belief that coffee disturbs sleep to actual wakefulness caused by caffeine*

Data are combined responses (exps. c and d) as in Table 4, broken down according to affirmative and negative beliefs about sleep disturbance by caffeine (Table 5).

Believe coffee disturbs?	How long to fall asleep?	Lactose nights	Caffeine nights
Yes	Longer time than usual	4	29
	Not longer than usual	44	23
No	Longer time than usual	11	24
	Not longer than usual	85	71

Analysis by Chi-square test. In the "Yes" group ($\chi^2 = 23.3$, $P < 0.01$) and also in the "No" group ($\chi^2 = 5.2$, $P < 0.05$), caffeine caused wakefulness, as compared with lactose. However, caffeine caused less wakefulness in the "No" group than in the "Yes" group ($\chi^2 = 12.3$, $P < 0.01$).

Table 7. *Relationship of morning headache to habitual caffeine intake*

Data are combined responses (exps. c and d) to "Did you have a headache before breakfast this morning?" In these experiments beverages were prohibited after lunch on experimental days. The question refers to the morning *following* ingestion of a sample containing lactose or 150 mg caffeine. Thus subjects who received lactose the previous evening would have abstained from caffeine for at least 18 hours at the time in question

Daily coffee (cups)	Headache?	Lactose nights	Caffeine nights
0	Yes	4	5
	No	47	43
1	Yes	2	0
	No	20	26
2–4	Yes	6	3
	No	43	41
5 or more	Yes	8	1
	No	24	35
Total	Yes	20	9
	No	134	145

Analysis by Chi-square test. For the entire group of responses the incidence of headaches was higher after lactose nights than after caffeine nights ($\chi^2 = 3.8$, $P < 0.05$). In the most habituated group, but not in any others, the incidence of headaches was significantly higher after lactose nights than after caffeine nights ($\chi^2 = 5.5$, $P < 0.05$). After lactose nights the incidence of headache was higher in the most habituated group than in the combined 0–1 cup group ($\chi^2 = 4.1$, $P < 0.05$)

In Table 6 we examine more generally the validity of subject's beliefs about whether or not coffee disturbs their sleep. The data show a highly significant difference in the incidence of wakefulness caused by caffeine, according to subjects' beliefs. More than half of the responses were positive (wakefulness) on caffeine nights in the group which asserted that coffee disturbed their sleep, whereas only one-quarter were positive in the other. On the other hand, even in the latter group, which believes coffee does not disturb their sleep, there was a significant effect of caffeine as compared with lactose.

Finally, Table 7 demonstrates the occurrence of caffeine withdrawal headache in the most habituated group on awakening after lactose nights (18 hours abstention from caffeine) but not after caffeine nights. Among those who hardly ever drink coffee the incidence of headache was equally low after caffeine and lactose nights.

Discussion

The experiments reported here show unequivocally that caffeine prolonged the time required for subjects to fall asleep. The effect occurred at both doses (150 and 200 mg) and regardless of whether the experiment purported to deal with hypnotic drugs or was frankly presented as an investigation into caffeine. The very striking effects of caffeine were particulary interesting in the former situation, where a natural reluctance to admit wakefulness might have been expected. In addition to causing wakefulness at bedtime, caffeine had a less prominent but still definite action of longer duration, manifested by a disturbance of the soundness of sleep.

A most interesting phenomenon was revealed when a known caffeine sample was given on the first night, followed by blind administration of caffeine or lactose on the succeeding four nights. The wakefulness produced by known caffeine was minimized in subjects' reports, so that effects appeared to be hardly greater than on lactose nights; yet the identical caffeine dose given to the same subjects in a blind experiment produced definite wakefulness. We are confronted with a "reverse placebo effect" of striking magnitude. We can only speculate that its cause here may lie in the sophistication and skeptical attitude of the medical student group, but it may well prove to be a phenomenon of more general occurrence.

In subjects who drink a great deal of coffee habitually, caffeine produced less wakefulness than in those who drink coffee only occasionally. It is tempting to surmise that this difference reflects an acquired tolerance to the drug. However, the alternative cannot be ruled out, that some subjects are (and always have been) intrinsically less sensitive to caffeine and that consequently they drink more coffee in order to obtain the

desired stimulation. We judge that it would be very difficult to secure sufficient cooperation from habituated subjects, to conduct an experiment involving a long period of abstinence followed by re-testing, since even the daily abstinence periods required here were regarded by them as a hardship. It would be interesting, but also difficult, to conduct experiments like those described here with adolescents who have not yet established the coffee-drinking habit, followed some years later by a survey to discover if the least sensitive individuals go on to drink the most coffee.

The finding that subjects, on the whole, recognize the sleep-disturbing effect of caffeine on themselves is not surprising. It is curious, however, that even among those who believe they are not disturbed by coffee taken at bedtime, there was a significant (though relatively small) caffeine effect.

The demonstration of caffeine withdrawal headache in the most habituated group represents the first experimental confirmation of this phenomenon under conditions of ordinary coffee-drinking. The artificial production of such headaches by DREISBACH and PFEIFFER (1943) required an ascending daily dosage, to very high levels before withdrawal. In our experiments the incidence of headache following a period of abstinence was relatively small, but nonetheless significant. Headache occurred in the morning, about 18 hours following the last previous ingestion of coffee, and could be prevented by a single dose of 150 mg caffeine at bedtime the previous night. Our findings are thus in accord with the experience commonly related by habituated coffee-drinkers who assert that breakfast coffee serves to ward off or cure morning headache.

An important finding in these experiments is the great variation between subjects in their sensitivity to caffeine-produced wakefulness (cf. Fig. 1). These individual differences will be examined more thoroughly in a subsequent communication.

Finally, we point out some important advantages in the choice of medical students as subjects for experiments in which criteria of drug effect are necessarily subjective. That the measures of drug response are crude cannot be denied; but this disadvantage is circumvented here by the rigidly controlled double-blind design. With ordinary subjects the ability to follow directions, the desire to do so, and the capacity to make accurate observations usually leave much to be desired, so that the validity of the data suffers and unnecessary sources of variation are introduced. Medical students, by virtue of their professional interest in the investigation are motivated to cooperate fully, and by virtue of their superior intelligence are able to produce accurate and carefully considered data. Moreover, the participation of an entire class precludes the pitfalls attendant upon the use of so-called "normal volunteers", who are

products of a particular self-selection process and often turn out to be wholly atypical of the population to which they belong (LASAGNA and VON FELSINGER 1954). The compositional stability of the medical student group also ensures that long-term studies can be carried out (even extending over several years) with the same subjects under reasonably reproducible circumstances and without serious depletion by drop-out.

Summary

Certain psychotropic effects of caffeine were investigated in double-blind placebo-controlled experiments with 230 medical students.

1. Caffeine prolonged the time required to fall asleep and disturbed the soundness of sleep when administered in a dose of 150 or 200 mg in decaffeinated coffee shortly before bedtime.

2. Caffeine caused distinctly less wakefulness in subjects who habitually drank a great deal of coffee.

3. Some subjects among those who drank most coffee manifested morning headache after about 18 hours abstinence and this headache was prevented by a single dose of 150 mg the previous evening.

4. An interesting "reverse placebo effect" was revealed, whereby subjects receiving known caffeine tended to minimize its sleep-disturbing effects.

5. Important advantages in the use of medical students as subjects in experiments of this kind are pointed out, quite apart from any didactic value of such exercises.

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LACK OF RELATIONSHIP BETWEEN GASTRIC CARCINOMA AND INTAKE OF BEVERAGES CONTAINING CAFFEINE

AVRAM GOLDSTEIN, M.D., AND RICHARD WARREN, M.D.

CANCER CELLS POSSESS the hereditary property of uncontrolled growth, and somatic mutation has been advanced as a possible cause of cancer. Caffeine is mutagenic in fungi,[3] bacteria,[4] and *Drosophila*.[1] A large part of the human race is exposed to caffeine in the form of coffee and tea daily throughout adult life. In bacteria, caffeine concentrations of 150 µg. per ml. were shown to be significantly mutagenic.[4] In man, each single cup of coffee containing about 130 mg. of caffeine would expose the cells of the gastric mucosa to about 600 µg. per ml. if the stomach were empty, or to about 60 µg. per ml. if the stomach were full. The absorption of caffeine is essentially complete within 1 hour. Following absorption, caffeine is distributed throughout body water so that all body tissues are then exposed during the several hours until metabolic conversion is complete, but at very much lower concentrations.[2] Thus, in coffee and tea drinkers, the cells of the gastric mucosa are exposed to a much higher caffeine concentration than are any other cells in the body.

The facts cited previously raise the question whether caffeine may be capable of causing somatic mutation and consequent carcinogenesis in the stomach. If so, then patients with gastric carcinoma might be expected to have had a higher daily caffeine intake than a group of comparable patients with cancer at another site, or than the comparable population of healthy adults. Accordingly, we surveyed the hospital records of 681 male patients with a diagnosis of gastric carcinoma and 311 male patients with a diagnosis of cancer of the colon. Of these records, 136 and 66 respectively showed both a confirmed diagnosis of carcinoma of stomach or colon and adequate information about intake of beverages containing caffeine. The results, presented here, indicate no relationship between gastric cancer and the consumption of caffeine.

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TABLE 1
ANALYSIS OF HOSPITAL RECORDS

Diagnosis	Tot. no. records examined	Records satisfying criteria No.	%
Gastric ca.			
Stanford-Lane	212	50	24
San Francisco	472	86	18
TOTAL	684	136	42
Colon ca.			
Stanford-Lane	94	26	28
San Francisco	217	40	18
TOTAL	311	66	46

METHODS AND RESULTS

We reviewed the hospital records of all male patients entering Stanford-Lane Hospital, San Francisco, Calif., between 1932 and 1958 and San Francisco County Hospital, San Francisco, Calif., between 1911 and 1957, under the diagnostic headings of "gastric carcinoma" and "carcinoma of the colon." For inclusion in the analysis a record had to meet 2 criteria. First, the diagnosis had to be confirmed by biopsy, autopsy, or exploratory laparotomy. Second, the record had to contain definite information about the intake of beverages containing caffeine, and such information had to be recorded prior to establishment of the diagnosis. We sought thus to avoid underestimation of the habitual long-term caffeine intake in patients with gastric cancer who might have reduced their coffee and tea drinking because of the gastric symptoms. Table 1 presents data on the total number of records reviewed at both hospitals and the number that met both criteria for inclusion.

Each patient included in the analysis was classified as to age (by decade) and also as to the number of cups of coffee or tea drunk daily. If the hospital record included a definite statement that coffee or tea was drunk, but no indication of the amount, the patient was classified under "unknown quantity." The data obtained from the 2 hospitals were remarkably similar and are therefore pooled in Tables 2

and 3. Table 4 presents the frequency distributions for caffeine intake irrespective of age in patients with cancer of the stomach and cancer of the colon and in the healthy population of the United States.

DISCUSSION

The data show the well-known difference in age distribution between patients with carcinoma of the stomach and those with carcinoma of the colon. The consumption of beverages containing caffeine was remarkably similar in 2 patient groups; the slightly lower frequency of heavy consumers in the colon cancer group probably reflects the common reduction of caffeine intake in elderly people.

TABLE 2
CARCINOMA OF THE STOMACH

Pt. age, yr.	No. cups bev. containing caffeine consumed daily					Tot. no. pt.
	0	1-2	3-4	5+	Unknown*	
≤40	0	2	1	1	2	6
41-50	1	4	4	5	5	19
51-60	4	13	7	6	5	35
61-70	1	13	7	5	8	34
>70	3	17	10	2	10	42
TOTAL	9	49	29	19	30	136

* The figures in this column indicate the numbers of patients who consumed beverages containing caffeine in unspecified amounts.

The frequency of total abstainers was almost identical in the 2 patient groups and in the United States population cross section. Unfortunately, the arbitrary categories of caffeine consumption used in the Roper survey[5] made exact comparisons impossible with respect to those who did drink beverages containing caffeine, however, frequencies in the 1 to 2 cup and the pooled 3 or more cup categories differ but little from the corresponding patient groups.

We recognize that the retrospective type of analysis employed here is subject to many uncertainties, and also that the validity of information about caffeine consumption obtained incidental to a general medical history may be questioned. Nevertheless, we see no good reason to suspect systematic bias as between the 2 patient groups. The good agreement between data obtained at 2 different hospitals also suggests a certain reliability of the findings.

It seems quite clear that the 2 patient groups do not differ in any important way with respect to long-term caffeine consumption. The hypothesis that caffeine plays any significant role in the genesis of gastric carcinoma is thereby rendered improbable.

TABLE 3
CARCINOMA OF THE COLON

Pt. age, yr.	No. cups bev. containing caffeine consumed daily					Tot. no. pt.
	0	1-2	3-4	5+	Unknown*	
≤40	0	0	1	0	1	2
41-50	0	1	0	0	1	2
51-60	1	5	5	1	1	13
61-70	2	9	5	4	4	22
>70	1	9	9	2	6	27
TOTAL	4	24	20	6	12	66

* The figures in this column indicate the numbers of patients who consumed beverages containing caffeine in unspecified amounts.

SUMMARY

Caffeine is known to be mutagenic in lower organisms. In coffee and tea drinkers, the gastric mucosa is exposed to caffeine concentrations in excess of those known to be mutagenic in bacteria. It is postulated that caffeine might cause gastric carcinoma by inducing somatic mutation. The hypothesis was tested by reviewing hospital records of male patients with gastric carcinoma and comparing their habitual daily caffeine consumption with that of a control group of male patients with carcinoma of the colon. One hundred and thirty

TABLE 4
COMPARISON OF CONSUMPTION OF BEVERAGES CONTAINING CAFFEINE IN PATIENTS WITH GASTRIC AND COLON CARCINOMA AND IN A CROSS SECTION OF THE ADULT POPULATION OF THE UNITED STATES

Population	Frequency distribution*				
	No. cups beverage containing caffeine consumed daily				
	0	1-2	3-4	5+	Unknown†
Gastric ca. pt.	7	36	21	14	22
Colon ca. pt.	6	36	30	9	18
Adult population	8	41	51		

*Frequency distributions are given as percentages of the total number in each group. The distributions for gastric and colon carcinoma are from Tables 1 and 3; that for the United States population is derived from a survey made by Roper[5] in 1947 on a stratified cross section of the population more than 14 years of age.

†This column includes patients who drank beverages containing caffeine in unspecified amounts.

six records of patients with gastric cancer and 66 of patients with cancer of the colon met the dual criteria of definitely established diagnosis, and adequate information about the intake of beverages containing caffeine.

The 2 groups of patients did not differ in any important way from each other or from the healthy United States population with respect to caffeine consumption. The hypothesis that caffeine plays any significant role in the etiology of gastric carcinoma can probably be rejected.

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CHAPTER

19 CENTRAL NERVOUS SYSTEM STIMULANTS

[*Continued*]

II. The Xanthines

J. Murdoch Ritchie

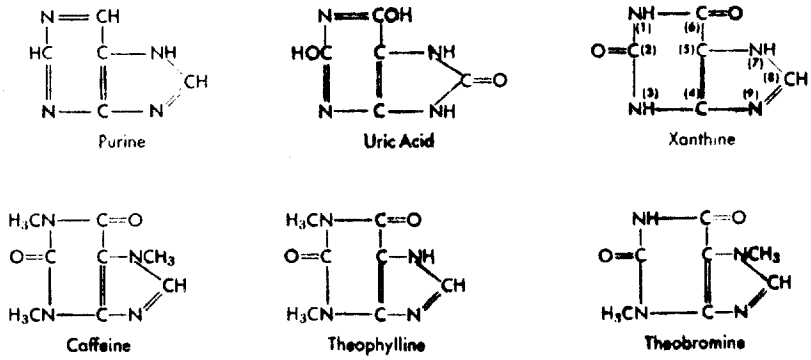
CAFFEINE, THEOPHYLLINE, AND THEOBROMINE

Source and History. Caffeine, theophylline, and theobromine are three closely related alkaloids that occur in plants widely distributed throughout the world. From earliest times, man has made beverages from aqueous extracts of these plants. *Coffee*, the seeds of *Coffea arabica* and related species, contains caffeine. *Tea*, the leaves of *Thea sinensis*, contains caffeine and theophylline. *Cocoa*, obtained from the seeds of *Theobroma cacao*, contains caffeine and theobromine. *Maté*, the national drink of many South American countries, contains caffeine. Even some so-called soft drinks, particularly the cola-flavored drinks popular in the United States, contain caffeine because they are made from the nuts of the tree, *Cola acuminata*. These kola nuts, the guru nuts chewed by the natives of the Sudan, contain about 2% caffeine.

The earliest history of these drinks is lost in considerable obscurity, the absence of fact being compensated by a profusion of conjectural statements and mythical stories. For example, legend credits the discovery of coffee to a prior of an Arabian convent. Shepherds reported that goats that had eaten the berries of the coffee plant gamboled and frisked about all through the night instead of sleeping. The prior, mindful of the long nights of prayer that he had to endure, instructed the shepherds to pick the berries so that he might make a beverage from them. The success of his experiment is obvious from the fierce opposition stirred up by the more orthodox section of the priests against the use of this devotional antisoporific, and from the popularity of coffee today. A billion kilograms are consumed annually in the United States alone.

Modern pharmacological studies of caffeine have amply confirmed the ancient belief that caffeine has a stimulant action, which is the basis for the popularity of all the caffeine-containing beverages. Caffeine and related compounds, however, possess other important pharmacological properties and are valuable therapeutic agents.

Chemistry. Caffeine, theophylline, and theobromine are methylated xanthines. They are often spoken of as *xanthine derivatives, methylxanthines*, or merely *xanthines*. Xanthine itself is dioxypurine and is structurally related to uric acid. Caffeine is 1,3,7-trimethylxanthine; theophylline, 1,3-dimethylxanthine; and theobromine, 3,7-dimethylxanthine. The structural formulas of purine, uric acid, xanthine, and the three pharmacologically important xanthine derivatives are as follows:



Studies of the actions of congeners of the methylxanthines have revealed structure-activity relationships of a complex nature (Scott *et al.*, 1946; Quimby *et al.*, 1958; Armitage *et al.*, 1961). Although these investigations are of great theoretical interest, no synthetic compound has yet been described that offers any therapeutic advantage over naturally occurring xanthines.

PHARMACOLOGICAL ACTIONS

Caffeine, theophylline, and theobromine share in common several pharmacological actions of therapeutic interest. They stimulate the central nervous system (CNS), act on the kidney to produce diuresis, stimulate cardiac muscle, and relax smooth muscle, notably bronchial muscle. Because the various xanthines differ markedly in the intensity of their actions on various structures, one particular xanthine is usually more suitable than another for any specific therapeutic effect and has fewer side actions. Table 19–1 depicts the relative potencies of the xanthines in producing various effects.

Central Nervous System. Caffeine is a powerful CNS stimulant; theophylline is less so; and theobromine is virtually inactive in this respect. Thus, caffeine is the xanthine usually employed clinically for its central action, although theophylline also has specific uses as a respiratory stimulant. Caffeine excites the CNS at all levels. The cortex is first affected and then the medulla, while the cord is stimulated only by very large amounts.

Cortex. Caffeine stimulates all portions of the cortex. Its main action is to produce a more rapid and clearer flow of thought, and to allay drowsiness and fatigue. After taking caffeine one is capable of a greater sustained intellectual effort and a more perfect association of ideas. There is also a keener appreciation of sensory stimuli, and reaction time to them is appreciably diminished. This accounts for the hyperesthesia, sometimes unpleasant, which some persons experience after drinking too much coffee. In addition, motor activity is increased; typists, for example, work faster and with fewer errors. However, recently acquired motor skill in a task involving delicate muscular coordination and accurate timing may not be improved (Goldstein *et al.*, 1965) or may even be adversely affected. These effects may be brought on by the administration of 150 to 250 mg of caffeine, the amount contained in 1 or 2 cups of coffee or tea.

Medulla. Caffeine also stimulates the medullary respiratory, vasomotor, and vagal centers. This stimulant action is of therapeutic value when the medulla is depressed by morphine and other drugs. High blood concentrations of caffeine are required. For example, a dose of caffeine of 150 to 250 mg taken orally produces little change in respiration; however, the same amount administered parenterally definitely stimulates the respiratory center. Caffeine increases the respiratory minute volume to a much greater extent in individuals breathing 3 or 5% carbon dioxide than in subjects breathing room air; this suggests that caffeine may act by sensitizing the respiratory center to carbon dioxide (Richmond, 1949). Parenterally administered theophylline also increases the rate and the depth of respiration, even in normal individuals. The stimulation of the vasomotor and vagal centers by xanthines can best be demonstrated in medullary perfusion experiments, because after systemic administration the peripheral effects of the drug on the circulation tend to mask the central actions (*see* below).

Table 19–1. RELATIVE PHARMACOLOGICAL ACTIVITY OF THE XANTHINES

XANTHINE	CNS AND RESPIRATORY STIMULATION	CARDIAC STIMULATION	CORONARY DILATATION	SMOOTH MUSCLE RELAXATION	SKELETAL MUSCLE STIMULATION	DIURESIS	PHOSPHODIESTERASE INHIBITION	
							Heart	*Brain*
Caffeine	1 *	3	3	3	1	3	2	1
Theophylline	2	1	1	1	2	1	1	1
Theobromine	3	2	2	2	3	2	2	—

* 1 = most active.

Cord. After the administration of large amounts of caffeine or theophylline, the entire CNS, including the spinal cord, is stimulated. Reflex excitability is increased, and lower motor centers may be directly excited. In experimental animals this may lead to clonic convulsions and death. However, in man the toxic dose of caffeine is so large (over 10 g) that human fatality is unlikely.

There is no doubt that the excitation of the CNS produced by large amounts of caffeine is followed by depression. There has been considerable controversy, however, as to whether this is also true after the mild physiological stimulation produced by the small amounts contained in the average cup of tea or coffee.

Cardiovascular System. The xanthines, especially theophylline, have important actions on the circulatory system of which therapeutic use is made. Even caffeine, which is the least active in this respect, has a powerful effect on the circulation, but its side effects on the CNS and on metabolism preclude its clinical use for cardiovascular effects.

All parts of the circulatory system are affected in some way by the xanthines. In several instances these various actions are antagonistic. Therefore, the observation of a single function, for example, the blood pressure, is deceiving because the drugs may act on a variety of circulatory factors in such a way that the blood pressure may remain essentially unchanged. The observations of Starr and coworkers (1937) on the cardiovascular effects of xanthines in human subjects are presented in Table 19–2.

Heart. The xanthines stimulate the myocardium directly. When they are added to the perfusion fluid of an isolated mammalian heart, the force of contraction, the rate, and the cardiac output increase; in experimental animals this inotropic effect is accompanied by an increase in duration of the action potential of atrial muscle (Gubareff and Sleator, 1965). When they are given to an intact animal, however, some of these effects may be masked because the xanthines, especially caffeine, stimulate the medullary vagal nuclei and thus tend to produce a decrease in heart rate. Consequently, as a result of these two opposing actions, there may be a slight bradycardia, a tachycardia, or no change at all in heart rate. After large doses of caffeine in both man and experimental animals, the direct stimulation of the myocardium predominates and a definite tachycardia ensues. Eventually this stimulation may become so great that it causes cardiac irregularities. Occasionally, arrhythmias are encountered in persons who use caffeine beverages to excess.

The direct myocardial action of the xanthines, particularly theophylline, causes an increase in cardiac output, which may occur even in the absence of tachycardia. There is an accompanying decrease in the venous

Table 19–2. CARDIOVASCULAR, RESPIRATORY, AND METABOLIC EFFECTS OF CAFFEINE AND THEOPHYLLINE IN HUMAN SUBJECTS UNDER BASAL CONDITIONS

FUNCTION	CAFFEINE AND SODIUM BENZOATE * (0.5 G, INTRAMUSCULARLY)		AMINOPHYLLINE † (0.48 G, INTRAMUSCULARLY)	
	Decrease %	*Increase %*	*Decrease %*	*Increase %*
Pulse rate	2.4		1.7	
Systolic pressure	4.0			6.8
Diastolic pressure	3.8			7.0
Cardiac output		14.8		25.8
Stroke volume		19.1		29.4
Left ventricular work per minute		9.3		35.4
Left ventricular work per beat		13.8		38.0
Heart volume	0.8		0.3	
Metabolic rate		6.9		7.8
Arteriovenous oxygen difference	6.1		12.4	
Respiratory rate	4.1			2.7
Respiratory volume		12.6		23.8
Peripheral circulatory resistance	14.3		18.9	

* Average results in four patients.

† Average results in seven patients.

(Data of Starr, Gamble, Margolies, Donal, Joseph, and Eagle, 1937. Courtesy of the *Journal of Clinical Investigation.*)

filling pressure, which is caused at least partly by the more efficient emptying of the heart. In normal individuals the lowering of filling pressure may outlast the stimulation of the myocardium, so that the rise in cardiac output is brief and may be followed by a fall below the initial level. In heart failure, however, the venous pressure is initially rather high; and the cardiac stimulation, together with the lowering of venous pressure, produced by theophylline leads to a marked increase in cardiac output that occurs almost immediately and persists for 30 minutes or more after intravenous administration (Howarth *et al.*, 1947; Fowell *et al.*, 1949). Thus, theophylline is a valuable drug in the emergency treatment of congestive heart failure.

Blood Vessels. Caffeine dilates the coronary, pulmonary, and general systemic blood vessels by a direct action on the vascular musculature. Caffeine also tends to constrict blood vessels by stimulating the medullary vasomotor center. After therapeutic doses, the peripheral vasodilator action predominates. Oncometric studies in animals show that there is a definite increase in organ volume following the administration of xanthines. Vasodilatation, coupled with an augmented cardiac output, results in an increased blood flow. However, in humans the increase in peripheral blood flow is short lived (Stewart and Jack, 1940) and probably is not of sufficient magnitude and duration to be of value in the treatment of peripheral vascular disease.

Cerebral Circulation. In contrast to their direct dilating effect upon the systemic blood vessels the xanthines cause a marked increase in cerebral vascular resistance with an accompanying decrease in cerebral blood flow and in the oxygen tension of the brain (Wechsler *et al.*, 1950; Moyer *et al.*, 1952a, 1952b). The xanthines probably act directly on the musculature of the cerebral arterioles. It is this vasoconstriction, rather than the decrease in cerebrospinal fluid pressure that may also occur, that is believed to be responsible for the striking relief of hypertensive headache by the xanthines.

Coronary Circulation. Experimentally, it can be demonstrated that the xanthines dilate coronary arteries and increase coronary blood flow. This has led to their extensive use in the treatment of coronary artery disease. However, opinion as to their value is so divided that the controversial evidence in this field will be presented in some detail.

Pharmacological studies show that the xanthines increase the diameter of isolated coronary arterial rings, increase the flow into or the outflow from the coronary arteries of perfused hearts, and increase the coronary flow in experimental animals *in situ* (*see* the extensive bibliographies in Boyer and Green, 1941; Wégria, 1951; and Charlier, 1961). However, because of the myocardial stimulant action of the xanthines, myocardial work, oxygen uptake, and the coronary A-V oxygen difference also increase. Therefore, in spite of the increase in coronary flow there may be no increase in coronary reserve. Indeed, Schmidt (1951) has concluded that the increase in coronary blood flow produced by aminophylline in dogs in no way compensates for the increased oxygen demands of the heart that result from the increased work it performs. Because of this, he has characterized aminophylline as "an undesirable or malignant vasodilator."

Studies of the effect of xanthines on experimental coronary occlusions have also provoked much controversy (*see* Russek, 1960; Charlier, 1961). In some studies on dogs, aminophylline promoted the development of collateral circulation to experimentally induced myocardial infarcts and lowered the mortality rate in experimental coronary occlusions. However, in similar studies on dogs and cats no beneficial effects of xanthines could be demonstrated; indeed, the incidence of ventricular tachycardia in cats was significantly higher in the treated than in the control group.

Thus, there is no *clear* indication from animal experiments that the xanthines may be useful clinically in the treatment of coronary artery disease. Nevertheless, xanthine therapy has received extensive clinical evaluation in patients with angina pectoris. There are numerous studies in man in which no benefit from xanthine therapy was observed. For example, Modell (1968) believes that the xanthines provide little for the relief or prevention of an attack and that there is little known about their pharmacological action on which to base such a hope. Some studies, however, on the comparative frequency and severity of attacks of angina pectoris in patients during exercise or hypoxia seem to support the claim for the clinical efficacy of the xanthines. Indeed, so favorable have been the results of his own studies that Russek (1960) believes that the unfavorable conclusions derived from animal experiments must be rejected as far as man is concerned. In his opinion the unimpressive responses to xanthine therapy in man are often the result of a failure to maintain an adequate concentration of theophylline in the blood, and he thinks that the xanthine derivatives should be reinstated as valuable agents in the treatment

of angina pectoris. The diverse claims have been reviewed by Charlier (1961).

There seems to be general agreement in the studies on man that under certain conditions the xanthines increase coronary blood flow. There is also abundant evidence that the drugs increase the work of the heart. The outstanding question is whether the blood supply to the myocardium increases to a greater extent than does the oxygen demand. Regardless of controversial claims and disappointing clinical results the xanthines will probably continue to be employed in the treatment of coronary insufficiency.

Blood Pressure. From the previous discussion it is obvious that the effect of xanthines on the systemic blood pressure is unpredictable. Both the central vasomotor and the direct myocardial stimulation favor an increase in blood pressure. Conversely, the central vagal stimulation and the peripheral vasodilatation favor a decrease. The end result of the opposing actions on blood pressure is not striking; usually there is a slight rise, seldom amounting to more than 10 mm Hg. The combination of vasodilatation and enhanced cardiac output raises the pulse pressure, produces a more rapid blood flow, and leads to a more efficient circulation. In the pulmonary circuit aminophylline causes a slight rise in pulmonary systolic and pulse pressure in unanesthetized dogs (Friedberg *et al.*, 1943). However, in anesthetized dogs aminophylline may reduce the pulmonary arterial pressure as a result of pulmonary vasodilatation (Quimby *et al.*, 1958). In man the pulmonary arterial pressure is usually also reduced.

Smooth Muscle. The xanthines also act on smooth muscle other than those of blood vessels. Their most important action in this respect is their ability to relax the smooth muscles of the *bronchi*, especially if the bronchi have been constricted either experimentally by histamine or clinically in asthma. Theophylline is the most effective and produces a definite increase in vital capacity. It is, therefore, of considerable therapeutic value in the treatment of bronchial asthma.

The xanthines may also overcome spasm of the biliary tract produced in man by the injection of morphine, dihydromorphinone, or codeine. The xanthines, particularly theophylline-containing compounds, have therefore been employed in the treatment of biliary colic.

The action of the xanthines on the motor activity of the *gastrointestinal tract* is unimportant therapeutically. Dilute solutions increase, and high concentrations depress, the tone and strength of contraction of isolated intestinal strips. The intravenous injection of aminophylline in man causes a transient suppression of motility in the large and small bowel.

Voluntary Muscle. The xanthines strengthen the contraction of isolated skeletal muscle elicited by both direct and indirect electrical stimulation, and render the muscle less susceptible to fatigue (Huidobro and Amenabar, 1945; Huidobro, 1945; Goffart and Ritchie, 1952).

The xanthines, particularly caffeine, increase man's capacity for muscular work; theobromine is the least effective. Because the increase in work performance is produced only by doses of caffeine that also cause subjective symptoms of central stimulation (Foltz *et al.*, 1942), it has been argued that the central effects of the xanthines account for the increased capacity for muscular work. Until evidence is produced to the contrary, however, the peripheral effects cannot be discounted.

Diuretic Actions. All the xanthines increase the production of urine. Theophylline is the most powerful xanthine diuretic, but its action is short in duration. Theobromine is less active, but its effect is more sustained. Caffeine is the least powerful. A more detailed analysis of the diuretic action is given in Chapter 39.

Gastric Secretion. The controversy as to whether caffeine has a significant effect on gastric secretion has been largely resolved by the experiment of Ivy and coworkers. Paradoxically, the dog, which faithfully mimics man in so many of its gastric responses, is not readily affected by caffeine. However, in cats and humans moderate doses of caffeine result in a prolonged augmentation of gastric secretion (Roth and

Ivy, 1944a). The combined action of caffeine and histamine is greater than the sum of their individual actions with respect to the secretion of both acid and pepsin (Grossman *et al.*, 1945). Moreover, a number of investigators have shown that the administration of caffeine to animals in large single doses, in smaller repeated daily doses, or by intramuscular injection in beeswax results in pathological changes in the gastrointestinal tract and ulcer formation (*see* Merendino *et al.*, 1945). The significance of experimental peptic ulcers produced in this way has been questioned because of the high doses used. However, in view of the responsiveness of the human gastric mucosa to caffeine, cognizance must be taken of the ubiquitous use of coffee and cola beverages in the pathogenesis of peptic ulcer, and in the management of the ulcer patient (Roth *et al.*, 1944; Roth and Ivy, 1944b).

The secretory response evoked by caffeine shows characteristic patterns in normal individuals, and in patients with gastric or duodenal ulcers. Moreover, the distinction is greater than that obtainable with an alcohol test meal. There is also evidence that individuals with a predisposition toward peptic ulcers, or patients with peptic ulcers who are in remission, exhibit an abnormal response (Musick *et al.*, 1946). These considerations have led Roth and coworkers to suggest that the use of a caffeine test meal may be of diagnostic and prognostic value (*see* Musick *et al.*, 1949; Roth, 1951; Littman *et al.*, 1955).

Blood Clotting. It has been known since 1920 that, following the administration of aminophylline to man, the coagulation time of the blood is shortened. The methylxanthines can also antagonize the *effects* of the fall in the concentration of circulating prothrombin normally caused by coumarin derivatives and can prevent the clotting defect associated with hepatic injury. The main action seems to result from an increase in the concentration of Ac-globulin (factor V) in the plasma (*see* Chapter 65), which, in turn, may be caused by an increase in the plasma concentration of free fatty acids produced by the xanthines (Bellet *et al.*, 1968); this action indirectly causes any circulating prothrombin to be more effective; there are also increases in the concentrations of circulating prothrombin and fibrinogen. Clinical studies in man, however, have failed to show that *therapeutic* doses of xanthines have a measurable effect on the coagulation time of the blood.

Metabolism. The xanthines, especially caffeine, cause a slight increase in the basal metabolic rate, which can be observed in habitual coffee drinkers. Numerous investigators have reported that the ingestion of 0.5 g of caffeine may increase the basal metabolic rate an average of 10% and occasionally 25%. The maximum change occurs from 1 to 3 hours after the administration of the drug. During the early phase, the medullary stimulation increases the respiratory minute volume and results in an excessive elimination of carbon dioxide. This produces a rise in respiratory quotient that is later followed by a compensatory fall (Haldi *et al.*, 1941).

Caffeine has certain actions on processes of intermediary metabolism. For example, it depresses the formation of urea from ammonium salts both in liver slices *in vitro* and in experimental animals (Bernheim and Bernheim, 1945). It also promotes creatinuria in man (Bachmann *et al.*, 1942).

Tolerance. An appreciable degree of tolerance may develop to certain effects of the xanthines, especially the diuretic and vasodilator actions. Cross-tolerance between the members of the group also occurs. Little or no tolerance develops to the central stimulation. Habituation is discussed under caffeine beverages.

Cellular Basis for the Action of the Xanthines. Early attempts to discover the cellular basis of the pharmacological actions described above soon revealed that the methylxanthines have pronounced effects on cellular metabolism. For example, when skeletal muscle is exposed to caffeine, large quantities of lactic acid are produced, the oxygen consumption is considerably increased, and there is a stronger muscle twitch; with high concentrations of caffeine, contracture results. Hartree and Hill (1924) showed that these effects are associated with a prolonged spontaneous liberation of heat that lasts until after the muscle has become completely inexcitable. They suggested that caffeine acts on skeletal muscle to release, slowly and continuously, the chemical processes normally induced by stimulation.

Sutherland and associates have more re-

cently revealed a biochemical mechanism that may underlie certain of the metabolic and physiological effects of methylxanthines (*see* Chapter 24). Having first established the critical role of cyclic 3′,5′-AMP in promoting glycogenolysis in certain tissues, they showed that the methylxanthines, particularly theophylline, are competitive inhibitors of cyclic nucleotide phosphodiesterase, an enzyme that catalyzes the conversion of cyclic 3′,5′-AMP to 5′-AMP (Butcher and Sutherland, 1962). Cyclic 3′,5′-AMP concentrations are thus elevated in some tissues following exposure to methylxanthines. The catecholamines (and several other hormones and neurohumors) also increase the concentration of cyclic 3′,5′-AMP in many tissues, but by the different mechanism of stimulation of cyclic 3′,5′-AMP formation (*see* Chapter 24). Thus, both the methylxanthines and the catecholamines would be expected to have those particular pharmacological properties that are thought to depend on their common ability to increase the cyclic 3′,5′-AMP concentration in the tissues. In addition, by virtue of their differing sites of action, these effects of methylxanthines could be expected to result in a potentiation of, rather than simple addition with, those effects of catecholamines that are due to increased concentrations of cyclic 3′,5′-AMP. Thus, the shared biochemical mechanism of action of xanthines and catecholamines may account for the fact that the xanthines markedly potentiate the cardiac inotropic responses to the catecholamines (Rall and West, 1963). The phosphodiesterase from cardiac muscle is more sensitive to theophylline than to caffeine (Butcher and Sutherland, 1962), whereas that from brain is equally affected by the two methylxanthines (Cheung, 1967); this suggests a partial biochemical basis for the differences in the relative sensitivity of heart and brain to the two drugs (*see* Table 19–1). Differences in penetration into brain may be another potential explanation for the greater activity of caffeine in this regard. However, there are few data at present to link the effects of methylxanthines on the CNS with the actions of cyclic 3′,5′-AMP.

These considerations seem to lay a plausible biochemical foundation not only for certain of the effects of xanthines on metabolic activity but also for their cardiac action, their bronchodilator action, and their actions on smooth and skeletal muscle (*see*, however, Weber, 1968, and McNeill *et al.*, 1969). The current literature is replete with studies on the interactions between numerous hormones and methylxanthines, with interpretations of the data centering primarily around the possible involvement of cyclic 3′,5′-AMP (*see* Sutherland *et al.*, 1968). While the methylxanthines have been of considerable value in the study of cyclic 3′,5′-AMP action, other effects of these compounds have been demonstrated and should be borne in mind.

Complementary studies, particularly on skeletal muscle, have emphasized the involvement of calcium in the pharmacological action of caffeine. When a frog muscle is exposed to high concentrations of caffeine (0.05%), a contracture develops. Unlike normal muscular contraction, this contracture is not accompanied by an electrical change in the membrane; there is neither a decrease in resting potential nor any marked increase in the transverse conductance of the muscle membrane (Axelsson and Thesleff, 1958). There is, however, a marked increase in both the influx and the efflux of calcium ions (Bianchi, 1961). When calcium is removed from the external medium, influx is prevented; however, caffeine still increases the calcium efflux (Bianchi, 1961) and still produces a contracture (Axelsson and Thesleff, 1958; Frank, 1960, 1963). For these reasons, it has been suggested that caffeine, which readily penetrates muscle cells, reduces the binding of calcium in both the membrane and the myoplasm (Bianchi, 1961, 1963), and causes a release of calcium from the sarcoplasmic reticulum (Weber and Hertz, 1968). The result would be an increase in the intracellular thermodynamic activity of calcium, which, in turn, would affect the contractile state of the muscle and would also account for the increase in the efflux of calcium.

It is interesting that local anesthetics block both the contracture produced by caffeine and the associated changes in the calcium fluxes. It has been suggested (Feinstein, 1963) that they do this primarily by prevent-

ing caffeine from releasing calcium from binding sites in the muscle fibers. The fact that caffeine rigor can be prevented by local anesthetics, but cannot be reversed once it has been induced, supports this suggestion.

The precise relationship between these experiments with calcium and the more biochemical experiments previously discussed is obscure and provides a fascinating field for further study.

Toxicology. A fatal dose of caffeine given to an animal produces convulsions because of the central stimulating effect. Early in the poisoning, these are epileptiform in nature; as the action of the drug on the spinal cord becomes manifest, strychnine-like convulsions may appear. Death results from respiratory failure.

In man the fatal oral dose of caffeine is estimated to be about 10 g, but death from overdosage is highly unlikely. Untoward reactions, however, may be observed following the ingestion of 1 g or more of caffeine. These are mainly referable to the central nervous and circulatory systems. Insomnia, restlessness, and excitement are the early symptoms, which may progress to mild delirium. Sensory disturbances such as ringing in the ears and flashes of light are common. The muscles become tense and tremulous. Tachycardia and extrasystoles are frequent, and respiration is quickened. The diuretic action may be prominent. The central symptoms of caffeine poisoning can be readily controlled by the administration of depressants of the CNS; the short-acting barbiturates are effective and convenient for this purpose.

Theophylline, on the other hand, can be quite toxic and has occasionally proved fatal. Aminophylline (theophylline ethylenediamine) must be injected slowly when given by the intravenous route; otherwise, headache, palpitation, dizziness, nausea, fall in blood pressure, and even precordial pain may occur. The wisdom of injecting xanthines intravenously in patients with severe myocardial damage has been questioned. However, the incidence of untoward responses appears to be very low provided adequate care is taken. The intramuscular injection of aminophylline is accompanied by local pain that may persist for many hours.

Toxic effects occur whatever the route of administration. For example, studies on children have shown that the same set of symptoms occur during severe intoxication with aminophylline, no matter whether the drug is given by the oral, rectal, or intravenous route. It is characterized by frequent vomiting, unusual thirst, and maniacal agitation leading in the severest cases to convulsions, shock, and death (Nolke, 1956; White and Daeschner, 1956).

Mutagenic Effects. Caffeine induces chromosomal breakage in the fruit fly, higher plants, and a variety of microorganisms. It has similar effects in man (Ostertag *et al.*, 1965). However, the drinking of tea or coffee does not increase the mutation rate much above the small natural rate, and so does not seem to present any significant toxic hazard in man.

Absorption, Fate, and Excretion. The xanthines are readily absorbed after oral, rectal, or parenteral administration. The rate at which they are absorbed depends on the xanthine preparation used (Schluger *et al.*, 1957) and on the route of administration (Truitt *et al.*, 1950; Waxler and Schack, 1950). The oral route is the most convenient, but absorption of the simple alkaloids from the gastrointestinal tract may be erratic, perhaps because of their poor aqueous solubility (about 2.0% for caffeine, 1.0% for theophylline, and less than 0.1% for theobromine). Furthermore, oral administration may give rise to gastric irritation, nausea, and vomiting. These considerations have led to the xanthines, especially theophylline, being given by rectal suppository or enema (*see* Ridolfo and Kohlstaedt, 1959); absorption, however, may still be slow and erratic. Another approach has been to use xanthine compounds that are both more soluble and less irritating. True salts (*e.g.*, choline theophyllinate) or one of the double salts that the xanthines form with a number of compounds (*e.g.*, caffeine and sodium benzoate) are employed. The most widely used theophylline compound is aminophylline, which is a combination of theophylline with ethylenediamine. The ethylenediamine seems to

be therapeutically inert, but its presence is important because it increases the amount of theophylline in solution about 20-fold.

The effect of the xanthines is greatest and most rapid when these complex salts are administered intravenously. Great care, however, must be exercised when they are administered by this route, especially to patients with severe myocardial damage, for high local concentrations may result in the precipitation of free alkaloids when salts are exposed to the pH of the blood. This may be responsible for the sharp, usually transient, fall in blood pressure that often occurs when injection is too rapid.

In the body the xanthines are only partially demethylated and oxidized. They are largely excreted as methyluric acids or as methylxanthines. Cornish and Christman (1957) found that in man theophylline is converted mainly to 1,3-dimethyluric acid; theobromine is excreted largely as both 3- and 7-methylxanthines; and caffeine is excreted as 1-methyluric acid and 1-methylxanthine in relatively equal amounts. About 10% of the alkaloids is excreted unchanged. Because none of the xanthines is completely demethylated, there is no increase in the excretion of true uric acid. This important fact means that the xanthines are not contraindicated in gout.

The enzymes responsible for the metabolic degradation of the methylated xanthines are not known, but xanthine oxidase is not involved (Brodie *et al.*, 1952; Bergmann and Dikstein, 1956).

Preparations. The xanthines are weakly basic alkaloids. They are usually dispensed in the form of soluble double salts that they form with a number of compounds. For oral administration either the free base or one of the double salts may be used; for parenteral administration, however, it is necessary to employ one of the salts.

Caffeine, U.S.P., also called theine, is a white crystalline, glistening substance soluble in water to the extent of 1:50.

Caffeine and Sodium Benzoate Injection, U.S.P., and *Citrated Caffeine*, N.F., are obtained by mixing caffeine with approximately equal parts of sodium benzoate and citric acid, respectively. They are white, rather bitter, water-soluble compounds. The former is the preparation of choice for hypodermic administration and is available in ampuls containing 500 mg/2 ml of solution for intramuscular injection. Caffeine citrate is available as official tablets in 60-mg size for oral use.

Theophylline, U.S.P., is a white crystalline, bitter powder that is only slightly soluble in water (1%). *Theophylline Tablets*, N.F., are available for oral use. *Elixophylline* is a preparation containing free theophylline dissolved in 20% ethanol. Oral doses are claimed to be rapidly absorbed and to produce little gastric irritation. Theophylline is also available as the loose complexes and double salts described below, the percentage of anhydrous theophylline by weight being indicated in parentheses after each heading.

Aminophylline, U.S.P. (*Theophylline Ethylenediamine*) (85%), is the most widely used of the soluble theophylline salts. It consists of a combination of approximately 85% anhydrous theophylline and 15% ethylenediamine. It occurs as white or slightly yellowish granules or powder with a slightly ammoniacal odor and a bitter taste; 1 g dissolves in about 5 ml of water. The following official (U.S.P.) preparations of aminophylline are available: solutions in ampuls that contain 250 mg/10 ml and 500 mg/20 ml, for intravenous administration; tablets in 100- and 200-mg sizes, for oral administration; rectal suppositories in 125-, 250-, and 500-mg sizes.

Theophylline Sodium Acetate, N.F. (60%), is available as a white crystalline powder soluble 1:25 in water and as tablets in 100- and 200-mg sizes.

Theophylline Sodium Glycinate, N.F. (51%), a white crystalline powder freely soluble in water, is claimed to produce little gastric irritation. Tablets, ampuls, suppositories, and elixirs are available.

Theophylline-meglumine (46%), also called GLUCOPHYLLINE, occurs as yellowish or white crystals freely soluble in water. Its action and therapeutic applications are the same as those of aminophylline. Suppositories in 500-mg size are available.

Oxtriphylline, N.F. (64%), also called *choline theophyllinate* and CHOLEDYL, differs from the preparations described above in that it is a true salt of theophylline in which the hydrogen atom at position 7 (*see* page 358) has been replaced by the choline cation. It is freely soluble in water to yield a solution of pH 9.7. Compared to aminophylline, it is more soluble, more stable, better absorbed from the gastrointestinal tract, and less irritating to the gastric mucosa. Tablets in 100- and 200-mg sizes are available.

Dyphylline, also called NEOTHYLLINE and LYFYLLIN, is 7-(2,3-dihydroxypropyl)theophylline. This neutral derivative of theophylline is stable in gastric juice. It produces less gastric irritation than most theophylline compounds. Tablets in 100- and 200-mg sizes, an elixir (100 mg/15 ml), and ampuls (500 mg/2 ml) are available.

Theobromine is available as the free alkaloid; the drug is also available as a variety of complexes containing roughly equimolecular proportions of theobromine and some other compound. These preparations are given only by the oral route. The

approximate percentage by weight of theobromine is indicated after each heading below.

Theobromine calcium salicylate (48%) (THEOCALCIN) is available as a white odorless powder that is only slightly soluble in water, and as tablets usually containing 500 mg of the complex.

Theobromine sodium acetate (60%) is a white crystalline, hygroscopic powder freely soluble in water. It is available as tablets (250 and 500 mg).

Theobromine sodium salicylate (56%), a freely soluble white powder, is available in 500-mg tablets.

THERAPEUTIC USES

The diverse pharmacological actions of the xanthines find many therapeutic applications. The drugs are of particular value for their effects on the *myocardium, smooth muscle, CNS,* and *kidney*. The preparations of caffeine are usually utilized only as central stimulants. Theobromine and theophylline are used interchangeably for their actions on the coronary circulation. Theophylline preparations, often aminophylline, are used to relax bronchial smooth muscle and to stimulate the myocardium.

Cardiac Uses. The direct myocardial stimulation produced by the xanthines is of considerable value in treating acute episodes associated with congestive heart failure. Their chief usefulness is for the relief of paroxysmal dyspnea associated with left heart failure. In this condition the immediate effects on cardiac output, venous pressure, and the bronchial muscle combine to afford rapid relief of pulmonary edema and dyspnea. Sole reliance is not placed upon the xanthines, and their use is combined with other conventional procedures, such as phlebotomy or the application of tourniquets to reduce the output of the right ventricle, morphine therapy to relieve dyspnea, and administration of oxygen to relieve hypoxia. The preparation of choice is aminophylline, which is given in the dose of 0.5 g by slow intravenous or by intramuscular injection. If indicated, this may be followed by intravenous digitalization. The action of the aminophylline is rapid in onset and lasts until that of the cardiac glycoside begins.

The xanthines have been employed to reduce the frequency and severity of attacks of *angina pectoris*, and to a more limited extent in the immediate management of coronary thrombosis. Obviously, in the face of a controversial literature no categorical statement as to the therapeutic efficacy can be made. Many physicians employ the xanthines with the attitude that, whereas they may do the patient no good, they can do him no harm. When xanthines are employed in the treatment of angina pectoris, they are given orally. The average single dose of aminophylline is 0.5 g. The average single dose of theophylline is 0.2 g given in a hydroalcoholic solution. In the treatment of coronary thrombosis aminophylline is given intramuscularly every 8 hours, in doses of 0.5 g each, until the patient is well enough to take medication by mouth.

Bronchial Asthma. Theophylline compounds, particularly aminophylline, play an important role in the management of the asthmatic patient. They are useful as prophylactic drugs, and are valuable adjuncts in the treatment of prolonged attacks and in the management of status asthmaticus.

Although ACTH and adrenocorticosteroids have provided new and effective weapons for the treatment of prolonged attacks of asthma, bronchodilator drugs still occupy an important place in therapy. The successful treatment of status asthmaticus calls for a variety of measures, including the use of oxygen or oxygen-helium mixtures, sympathomimetic drugs, expectorants, sedatives, and bronchial aspiration (*see* Barach, 1951). One of the most effective bronchodilating agents is aminophylline. For treatment of intractable bronchial asthma, aminophylline is first given by intravenous injection in a dose of 0.5 g. A high percentage of patients experience almost immediate relief for a period of time. It is necessary, however, to continue therapy in order to prevent recurrence. For this purpose, aminophylline can be given rectally either as a suppository containing 0.5 g, or more effectively as a retention enema containing 0.5 to 0.7 g in 20 ml of tap water. Rectal instillations are given morning and night for at least a week. When this therapy is combined with appropriate ancillary measures, prolonged periods of remission can sometimes be achieved.

Aminophylline, or some other theophylline compound, may be given over prolonged periods by mouth to reduce the number and severity of asthmatic attacks. The drug may be combined with ephedrine, which acts additively; a barbiturate may also be incorporated to counteract the combined central stimulant actions of the xanthine and the sympathomimetic. A single dose of 0.25 g is repeated three or four times daily.

Central Nervous System. The central action of the xanthines, particularly caffeine, is useful in treating cases of poisoning by central depressants. In morphine poisoning, the direct effect of caffeine on the medulla is of particular value. The drug is given by intramuscular injection, usually as caffeine and sodium benzoate (0.5 to 1.0 g).

Kidney. The use of xanthines as diuretics is discussed in Chapter 39.

Miscellaneous Uses. The intravenous injection of aminophylline has been shown to be effective in relieving the pain of acute biliary colic. The promising results obtained by clinical trial (Gladstone and Goodman, 1944) encourage the more extensive use of this compound for its antispasmodic action on the biliary passages. The dose is 0.25 to 0.5 g given slowly by the intravenous route.

The xanthines are widely employed [illegible]

ment of *headache*. It is now believed that they act as the result of a direct vasoconstrictor effect on cerebral blood vessels (*see* page 361). Caffeine, the preparation most commonly used, is seldom employed alone; rather, it is used in combination with an analgesic, such as a salicylate, in the treatment of ordinary types of headache, and in combination with an ergot alkaloid in the treatment of migraine. It is of interest that the withdrawal of caffeine from individuals habituated to it may also result in headaches. This "caffeine-withdrawal" headache can be relieved by the administration of caffeine. It is not unlikely that the incidence of this type of headache is high and provides a further explanation for the benefit obtained from the use of caffeine in analgesic mixtures.

XANTHINE BEVERAGES

The most popular xanthine beverages are coffee, tea, cocoa, and cola-flavored drinks. Coffee and tea contain caffeine, whereas cocoa contains theobromine. The caffeine content of tea leaves (about 2.0%) is higher than that of coffee beans (0.7 to 2.0%), but the beverages as finally prepared contain about equal amounts of the alkaloid. A 12-oz bottle of a cola drink contains 35 to 55 mg of caffeine. The average cup of coffee contains between 100 and 150 mg of caffeine, approximately a therapeutic dose. Naturally, the daily ingestion of even this amount of a potent alkaloid is bound to exert some pharmacological action. In addition to alkaloids, coffee contains certain oils, and there is an appreciable amount of tannin in tea.

There is little doubt that the popularity of the xanthine beverages depends on their stimulant action, although most people are unaware of any stimulation. The degree to which an individual is stimulated by a given amount of caffeine varies. For example, some persons boast of their ability to drink several cups of coffee in the evening and yet "sleep like a log." On the other hand, there are rare persons who are so sensitive to caffeine that even a single cup of coffee will cause a response bordering on the toxic.

The xanthine beverages present a medical problem in that they are a dietary source of a stimulant of the CNS. Often the physician is faced with the question whether to deny caffeine-containing beverages to patients. Children are more susceptible than adults to excitation by xanthines. For this reason, tea and coffee should be excluded from their diet. Even cocoa is of doubtful value. It has a high tannin content and, although the predominant alkaloid is theobromine, the caffeine content may be as high as 50 mg per cup.

The methylated xanthines are not excreted as uric acid and thus do not increase the uric acid content of the urine. Rather, they are excreted as methylxanthines and methyluric acids whose solubility is greater than that of uric acid. Furthermore, it is doubtful whether the amount of methyluric acid that is formed from the caffeine in xanthine beverages is large enough to be serious or indeed whether methylurates are deposited in gouty tophi (Weiss and Segaloff, 1959). The xanthine beverages, therefore, do not seem to be contraindicated in gout.

A patient with an active peptic ulcer should restrict his intake of caffeine-containing beverages. Even decaffeinated coffee stimulates gastric secretion to a limited extent because of other constituents of the bean. Individuals with peptic ulcer should consume their coffee (if at all) during meals, well diluted with cream because the buffering capacity of coffee is negligible.

Xanthine beverages are often denied to individuals with hypertension because of the action on the cardiovascular and nervous systems. Obviously, in a disease in which sedation is an important factor, stimulation by a xanthine is not to be desired. However, most authorities take the attitude that the complete denial of coffee to an individual who enjoys it is apt to be more disturbing than any stimulation he may receive from the beverage. Many patients with hypertension experience neither an increase in blood pressure nor nervousness from xanthine beverages. Indeed, theobromine is sometimes prescribed for its renal and, perhaps, coronary effects in such patients. Xanthine beverages accomplish the same end and, in addition, may relieve the headache so common in this syndrome. Therefore, coffee and tea are not denied, but their intake may have to be controlled.

Overindulgence in xanthine beverages may lead to a condition that might be considered one of chronic poisoning. Central nervous stimulation results in restlessness and disturbed sleep; myocardial stimulation is reflected in cardiac irregularities, especially premature systoles, and in palpitation and tachycardia. The essential oils of coffee may cause some gastrointestinal irritation, and diarrhea is a common symptom. The high tannin content of tea, on the other hand, is apt to cause constipation.

There is no doubt that a certain degree of tolerance (Colton *et al.*, 1968) and of psychic dependence (*i.e.*, habituation) develops to the xanthine beverages. This is probably true even in those individuals who do not partake to excess. However, the morning cup of coffee is so much a part of American dietary habit that one seldom looks upon its consumption as a drug habit, and there is no evidence that the practice is in any way harmful. The feeling of well-being and the increased performance it affords, although possibly obtained at the expense of decreased efficiency later in the day, are experiences that few individuals would care to give up.

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Determination of Caffeine in Plasma by Gas Chromatography

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A gas chromatographic method has been developed for the rapid, precise, and specific determination of caffeine in plasma. The method overcomes the major drawbacks of previous methods for the determination of caffeine in body fluids, which were: difficult isolation from interfering materials, a substantial blank error and low sensitivity. A standard response curve relating the signal-height ratio of caffeine to that of an internal standard, hexobarbital, permits quantitation of the amount of caffeine present. The method involves extraction of caffeine from plasma with chloroform, after the aqueous phase was adjusted to pH 11.5–12.0. The chloroform extract was evaporated to dryness and the sample was redissolved in carbon disulfide. Two milliliters of plasma was used and caffeine was determined at a concentration of 0.25 mcg./ml.

CAFFEINE, due to its widespread occurrence in beverages, is probably the drug consumed most today. There is, however, no rapid and sensitive method available for measuring this drug in biological fluids. Connors (1) reviewed many methods for the detection of xanthines, but these were generally not specific for caffeine. The older methods involved extraction of caffeine followed by a gravimetric or volumetric assay (4). These were succeeded by the colorimetric murexide reaction of Tanaka and Ohkubo (5). In addition a number of reagents that form colors have been employed by the authors and by others in an attempt to develop more specific tests for the xanthines. These were used in conjunction with paper (1–3, 13) and thin-layer chromatography (14). The reagents include mercuric chloride, potassium ferrocyanide, mercuric acetate, Dragendorff's reagent, and an alkaline phosphotungstate reagent. Visualization of the spots has also been achieved under UV light at 254 mμ using fluorescent thin-layer plates (10, 12, 17). Other methods include argentimetric (2), iodometric (1), solvent extraction (2, 8), ion exchange (2, 12), nonaqueous titrations (1), Kjeldahl (1), and spectrophotometric (2, 6–9, 11, 14, 15) methods. These have proved successful in measuring qualitatively, and in some cases

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quantitatively, pure xanthines. They were used in detecting mixtures of some xanthines and xanthines mixed with a number of other drugs.

Previously, the analytical method of choice in determining caffeine in biological systems has been spectrophotometry. The original development was made by Ishler *et al.* (6) and since then most of the determinations of caffeine in blood, tissue, and urine were based on this approach (2, 7–9, 21–23). However, these methods do not possess the sensitivity or specificity required for estimating concentrations of this drug produced by the ingestion of normal amounts of caffeine-containing beverages (2, 8, 21, 23). The authors have attempted to use a spectrophotometric assay but meaningful results could not be obtained at the concentration range of interest. The blank values were too high (relative to caffeine blood levels) and variable.

Gas chromatographic assays have been reported for many drugs in biological fluids (16), and xanthines have been examined in a number of GC systems (18, 19, 24–29). Such systems combine separation and quantitation of components and appear to offer distinct advantages over other methods of analysis.

EXPERIMENTAL

Equipment—A Varian Aerograph series 1200 gas chromatograph with a flame-ionization detector and a Varian model 20 recorder set at 20 in. hr.$^{-1}$ was used. The column employed was a 1.9 m. (6 ft.) stainless steel helix, 0.32 cm. (1/8 in.) o.d. packed with 3% OV 17 on Chromosorb W 100–120 mesh AW-DMCS H.P. (Applied Science lot 4223). The N_2 gas flow rate was 30 ml./min. Operating temperatures were: oven, 200°; injection port, 260°; and detector, 260°. Attenuations from 0.1 × 8 to 0.1 × 64 were used.

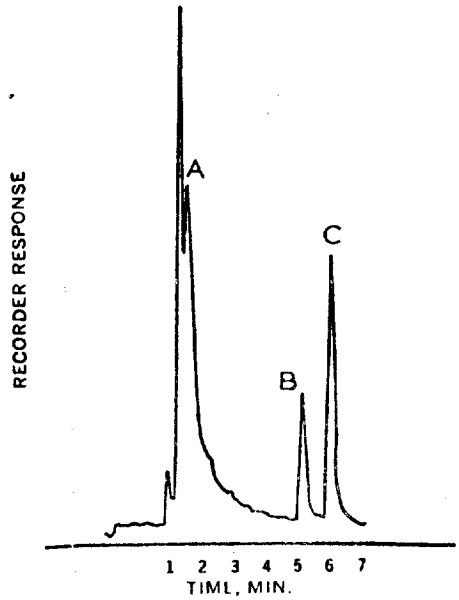

Fig. 1—Typical chromatogram. Key: A, carbon disulfide; B, hexobarbital (R_t = *4.3*)*; C, caffeine* (R_t = *5.2*)*. Column 3% OV 17.*

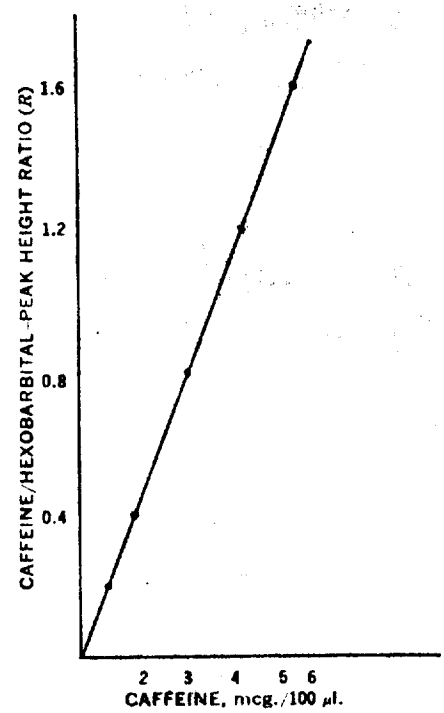

Fig. 2—Standard gas chromatogram response curve caffeine; internal marker, hexobarbital.

Reagents—(*a*) Caffeine (Eastman Organic) purified by vacuum sublimation; (*b*) hexobarbital (Winthrop), the internal standard; (*c*) reagent grade carbon disulfide (MCB) further purified by distillation; (*d*) heparin solution, 1,000 units/ml. (free of preservatives); (*e*) 2.5 *N* sodium hydroxide (Fisher Certified Reagent); (*f*) technical grade chloroform (Baker) freshly distilled.

Standard Curve—Standard curves were obtained by preparing solutions of caffeine 0.75–6.0 mcg./100 µl. and hexobarbital 2.0 mcg./100 µl. in carbon disulfide (20). Both caffeine and hexobarbital give sharp peaks with some slight tailing (Fig. 1). The standard curve given by the peak-height ratio of caffeine/hexobarbital *versus* caffeine concentration was linear and reproducible (Fig. 2).

Preparation of Plasma Samples—A 5-ml. sample of blood was taken, added to 2 drops of heparin, and spun down in a centrifuge (IEC International Clinical Centrifuge) for 10 min. at 2500–3000 r.p.m. Two milliliters of plasma was pipeted into a 25-ml. round-bottom screw-cap Pyrex tube with a Teflon lined cap. The pH was adjusted to 11.5–12.0 with 2.5 *N* sodium hydroxide. To this 10 ml. of chloroform was added. The tubes were then agitated for 15 min. and then centrifuged (IEC International Centrifuge series 2) for 10 min. at 1,000 r.p.m. Using a 10-ml. hypodermic syringe with a 10 cm. (4 in.) cannula, 75% of the chloroform layer was withdrawn and placed into a conical ground-glass

stoppered 15-ml. centrifuge tube. The chloroform extract was then evaporated to dryness in a 60–80° water bath, mounted on a steam cone. Bumping was prevented by placing an antibumping chip, previously boiled in carbon disulfide, into the tube, just prior to driving off the chloroform. One milliliter of hexobarbital solution (2 mcg./ml.) in carbon disulfide was pipeted into the tube. This solution was then concentrated to approximately 100 μl. by heating in the water bath at 40–60°. A 2-μl. sample of this concentrate was then injected onto the column.

RESULTS AND DISCUSSION

Prior to using the present assay to determine plasma levels of caffeine after the ingestion of a cup of coffee, attempts were made to measure the concentration of a known and unknown amount of caffeine in plasma. An accurately measured amount of caffeine was added to two plasma samples, one containing an unknown amount of caffeine and the other containing no caffeine. The plasma samples were treated as described under *Experimental*. A linear plot was obtained for both plasma samples when the caffeine/hexobarbital-peak height ratio was plotted against caffeine concentration (Fig. 3). Theoretically both graphs should be parallel, differing only in the intercept with the *y* axis. This was the case. The intercept was equal to the concentration of caffeine present in the plasma samples before the additions were made. The sample which contained no caffeine originally passes through the origin, while the other intercepts the *y* axis at a peak-height ratio of 0.2. This corresponds to a caffeine concentration of 0.5 mcg./ml.

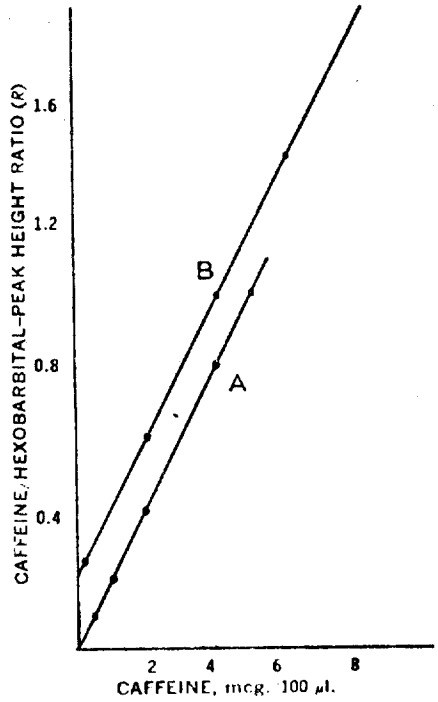

Fig. 3—Calibration curve of caffeine extracted from 2 ml. of plasma. Key: A, no caffeine present in original plasma sample; B, unknown amount of caffeine present in original plasma sample.

TABLE I—RECOVERY OF CAFFEINE ADDED TO PLASMA

Caffeine Added, mcg./ml.	Caffeine Found, mcg./ml.	Recovery, %
0.0	0.00	—
0.5	0.49	98
1.0	1.01	101
2.0	1.95	98
2.5	2.56	102

The standard curve (Fig. 2) can be compared to the calibration curve (Fig. 3A), since the two graphs share a common abscissa. The slope of the latter is 75% of the former. Taking into account that a 75% aliquot of the plasma extract was withdrawn, the values at each level of caffeine are identical for both curves. This indicates that the caffeine was quantitatively extracted (Table I).

The above procedure was repeated in five other subjects who had abstained from caffeine-containing beverages for 36 hr. Plasma samples were obtained and a calibration curve was determined for each. The curves passed through the origin and possessed the same slope. This indicates that the method is reliable and may be used for estimating caffeine plasma levels.

Absorption of Caffeine—Using the assay procedure, the absorption of caffeine, following the ingestion by three subjects of coffee containing 100 mg. of the drug, was studied. Caffeine was detected within 5 min. after ingestion. Peak plasma levels of 1.5–1.8 mcg./ml. were seen 50–75 min. after the dose. It was found that absorption could be described by a pseudo first-order process. The intersubject variation in the half-life of absorption was 8–17 min., while the intrasubject variation was much smaller. Apparent metabolic half-lives varied from 3.5–6 hr. in two subjects and from 6–10.5 in a third (*e.g.*, Fig. 4). Detailed data on the absorption and metabolism of caffeine in the body will be reported in a subsequent paper.

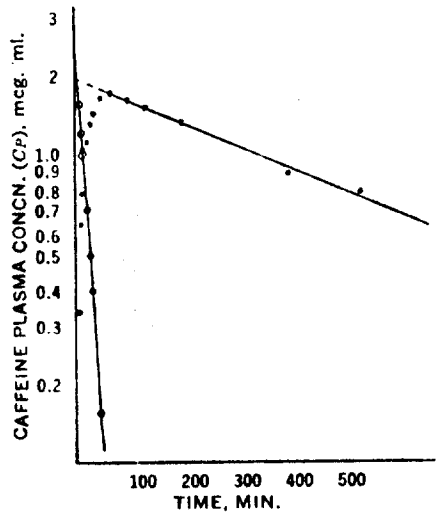

Fig. 4—Caffeine concentration after the ingestion of coffee containing 100 mg. of the drug. A graphical estimation of the metabolic and absorption (from the residual plot) half-lives are shown. Key: ●, observed data, $t_{1/2}^{m}$ = 6.0 hr.; ○, residual data, $t_{1/2}^{a}$ = 10.4 min.

SUMMARY

A gas chromatographic method has been developed for the determination of caffeine in plasma. A standard response curve relating signal-height ratios of caffeine to hexobarbital permits a quantitative estimation of the amount of caffeine present. The method is more rapid and sensitive than any previously reported. Plasma levels of caffeine after the ingestion of one cup of coffee have been studied and concentrations down to 0.25 mcg./ml. have been accurately determined.

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Keyphrases

Caffeine in plasma–analysis
Plasma analysis–caffeine
GLC–analysis
Hexobarbital–internal standard